TECHNIQUES AND INSTRUMENTATION IN ANALYTICAL CHEMISTRY

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EVALUATION
OF ANALYTICAL METHODS
IN BIOLOGICAL SYSTEMS

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PART C

DETERMINATION OF BETA-BLOCKERS IN BIOLOGICAL MATERIAL

edited by

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XII

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PREFACE

Evaluation of Analytical Methods in Biological Systems is a
problem-oriented series and the keyword associated with it is
"evaluation". For the evaluation of such a multi-dimensional field
as the determination of about fifty drugs by nine methods (spec-
trophotometry, spectrofluorometry, gas-liquid chromatography,
high--performance liquid chromatography, thin-layer chromato-
graphy, radioimmunoassay, enzyme immunoassay, fluoroimmunoassay
and radioreceptor assay), complete information is inevitably nece-
ssary. Therefore the main aim of the book is to provide a compre-
hensive coverage of the theory and practice of the determination
of beta-blockers in biological material. If this aim has been
achieved, the reader will have available a valuable source of
information for the evaluation of the methods from the point of
view of his or her actual needs - the drug and the analytical
problem. In addition the author(s) of each chapter were requested
to approach the problem very critically in evaluating the possibi-
lities of each method for the determination of beta-blockers in
biological material.

The book considers fifty beta-blockers (some more and some
less closely) and nine methods. The methods are divided into three
well known groups: (a) optical methods, (b) chromatographic me-
thods and (c) saturation methods. However, not only methods them-
selves are treated. The beginning of almost all determinations is
connected with the pre-analytical manipulation of samples, and
these problems are dealt with at the beginning of the book. The
output of every determination presents information relevant to the
user - the pharmacologist, toxicologist or physician, and the
quality of this information is closely connected with the under-
standing of the user s needs. These needs, in turn, should reflect
the possibilities of the analyst and the methods available. For a
better understanding, one chapter is devoted also to the pharmaco-
logist and one to the pharmacokineticist.

Recent evidence points to the increased importance of distin-
guishing optical isomers of drugs, especially beta-blockers, from
the point of view of their pharmacodynamics in addition to their
pharmacokinetics. In the light of this trend, one chapter on the

determination of optical isomers of beta-blockers in biological material is included.

The supplements, which conclude the book, contain a list of retention indices and a list of structures of beta-blockers.

It is hoped that analysts working on the determination of beta-blockers, whatever their level of experience, will find something of value in this book.

The Editor gratefully acknowledges the efforts of the contributors. He is indebted to Professor Rokus A. de Zeeuw for his confidence and for the help given during the preparation of the book and to Dr. M. Kouřilová, whose contribution to the book was much broader than the primary task of supervision of the English.

Bratislava   Vladimír Marko
September, 1988

INTRODUCTION

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Beta-adrenoceptor antagonists, or more familiar by beta--blockers, have undoubtely belonged to the most widely studied drugs over the past two decades. During this period, several thousand papers have been written on various aspects of their chemistry, biochemistry, pharmacology and clinical value. In 1982, the 20th anniversary of the discovery of the first beta-blocker, pronethanol, there were 23 beta-blockers on the market, 23 under clinical trials and 60 under experimental investigation (ref. 1). Beta-blockers marketed in 1987 are shown in Table 1. Their clinical uses range from angina pectoris, hypertension and tachycardia to the control af migraine, chronic alcoholism, schizophrenia, essential tremor and the cardiac effects of cocaine overdose, to mention only a few.

TABLE 1

Recently marketed beta-blockers

Acebutolol	Carteolol	Pindolol
Alprenolol	Indenolol	Propranolol
Atenolol	Labetalol	Sotalol
Befunolol	Levobunolol	Timolol
Betaxolol	Mepindolol	
Bisoprolol	Metipranolol	
Bucumolol	Metoprolol	
Bufetolol	Nadolol	
Bunitrolol	Nifenalol	
Bupranolol	Oxprenolol	
Carazolol	Penbutolol	

The development of beta-blockers can be traced back to 1905, when Langley was the first to suggest various inhibitory and excitatory receptor substances (ref. 2.). In addition, there are five other years that could be regarded as milestones in the history of beta-blockers. The first is 1906, when Dale applied the receptor concept to the adrenergic system (ref. 3). In 1948, Ahlquist published his classical paper describing the effects of six sympathetic stimulating compouds on a variety of adrenergic responses, and thus introduced the alpha- and beta-receptor concept (ref. 4). Ten years after Ahlquist's paper, in 1958 Moran and Perkins (ref. 5) and Powell and Slater (ref. 6) discovered and classified dichloroisoprenaline as the first beta-blocker. The first commercially available beta-adrenoceptor blocking drug was pronethanol, introduced by Black and Stephenson in 1962 (ref. 7). The last year in this series is 1964, when pronethanol was replaced with propranolol and when this drug was found to be effective against angina pectoris, cardiac rhythm disturbances and hypertension.

The above six milestones may be completed, from the point of view of this book, by a seventh, i.e. 1965, when the first papers reporting methods for the determination of beta-blockers appeared. This occured three years after the discovery of pronethanol, and these methods concerned pronethanol itself, propranolol (ref. 8) and toliprolol (ref. 9).

Of the thousands of papers on beta-blockers that have appeared since that time, about four hundred have dealt with methods for the determination of these drugs in biological material. They have described the determination of about 50 beta--blockers by nine methods - UV-VIS spectrophotometry, fluorescence spectrophotometry, gas-liquid chromatography, liquid or high--performance liquid chromatography, thin-layer chromatography, radioimmunoassay, enzyme immunoassay, fluorescence immunoassay and radioreceptor assay. Similarly to the other drugs (ref. 10), also in the determination of beta-blockers the greatest attention has been paid to chromatographic methods (about three quarters of all papers) with a prevalence of GLC and HPLC.

The historical development of methods for the determination of beta-blockers has been closely connected with the development of the analytical techniques themselves. This is illustrated in Fig. 1, where this development is expressed as the number of papers devoted to the different methods for the determination of

beta-blockers from 1965 to the mid 1980s. The first studies uti-
lized the optical properties of the drugs for their analysis, with
specrophotometry and spectrofluorometry being the main methods.
The 1970s were the years of GLC for beta-blockers and the 1980s
are characterized by a spectacular rise in the high-performance
liquid chromatographic method.
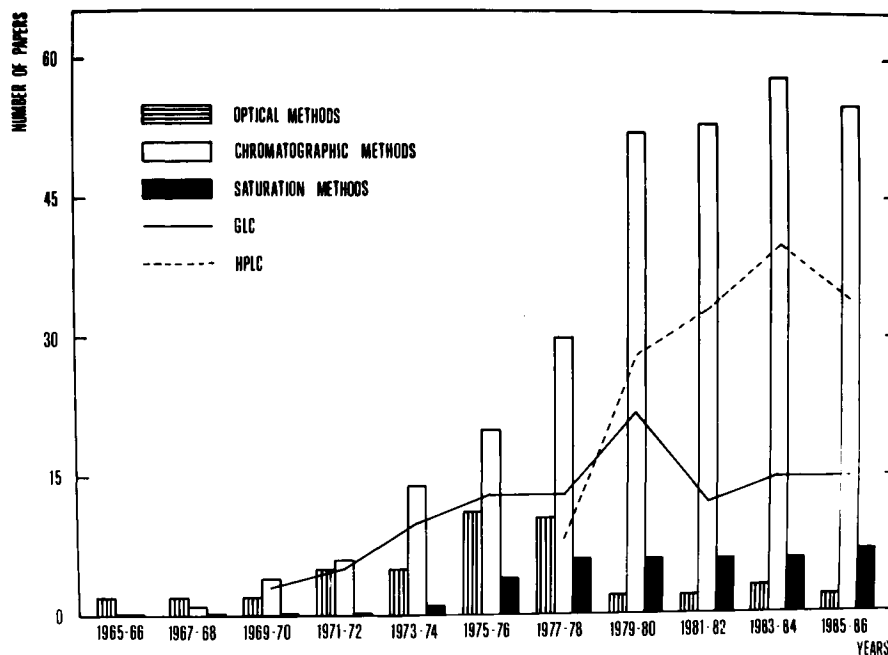
Fig. 1. Historical development of methods for the determination of
beta-blockers expressed as the number of papers devoted to these
methods.

    The preceding pages are the best answer to the question - Why
this book?
    The second question which could arise is - Why now? The data
in Fig. 1 might best answer this question. The decreasing number
of papers indicates that the development of the methods is almost
finished and no substantial changes are to be expected. The future
will be a time of utilizing the developed methods. Hence now is
the best time to summarize the knowledge on this topic.

4

REFERENCES

1 L.H. Smith, J. Chem. Technol. Biotechnol., 32 (1982) 433.
2 J.N. Langley, J. Physiol. (London), 33 (1905) 374.
3 H.H. Dale, J. Physiol. (London), 34 (1906) 163.
4 R.P. Ahlquist, Amer J. Physiol., 153 (1948) 586.
5 N.C. Moran and M.E. Perkins, J. Pharmacol. Exp. Ther., 123
   (1958) 233.
6 C.E. Powell and I.H. Slater, J. Pharmacol. Exp. Ther., 122
   (1958) 480.
7 J.W. Black and J.S. Stephenson, Lancet 2 (1962) 311.
8 J.W. Black, W.A.M. Duncan and G.G. Shanks, Brit. J. Pharmacol.
   Chemother., 25 (1965) 577.
9 K. Stock and E. Westermann, Biochem. Pharmacol., 14 (1965) 227.
10 M. Lesne, J. Pharmaceut. Biomed. Anal., 1 (1983) 415.

Chapter 1

RECENT DEVELOPMENTS IN THE CLINICAL PHARMACOLOGY OF β-BLOCKERS

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1.1 INTRODUCTION

In a classical experiment nearly forty years ago Ahlquist (ref.1) postulated that receptors were of two types: α-receptors, whose stimulation produced excitatory response, and β-receptors, whose activation resulted in inhibitory responses. Ten years later Powell and Slater (ref.2) reported that dichloroisoprenaline appeared to selectively inhibit responses mediated by β-receptors. Shortly after it was found that drugs of this type, in this case pronethalol, appeared to be clinically beneficial in the treatment of both angina pectoris (ref.3) and in hypertension (ref.4). Neither dichloroisoprenaline (DCI) or pronethalol were ever used widely; DCI because of significant β-receptor agonist activity and pronethalol because of evidence that it produced cancer in mice (ref.5). Since 1963 many more β-receptor antagonists have been developed and each year new drugs are introduced onto the market. Although these drugs share the common property of receptor antagonism they may differ from one another in relation to other pharmacodynamic properties such as cardioselectivity, partial agonist activity, and membrane stabilizing activity and in their pharmacokinetic properties.

All β-blockers share the common property of competitively inhibiting the effects of catecholamines at β-receptors (ref.6). Propranolol was the first one to be used clinically and is still the most widely used. It is a potent, non-selective β-antagonist with no partial agonist activity (also known as intrinsic sympathomimetic activity). However because of its non-selective β-antagonistic activity it blocks β-receptors in bronchial smooth muscle and skeletal muscle, thus interfering with bronchodilatation produced by epinephrine and other sympathomimetic amines and with glycogenolysis, which normally occurs during hypoglycemia. Propranolol is not therefore customarily used in asthmatic patients and should probably not be used in diabetics who are receiving insulin or oral hypoglycemic

agents. Over the last decade a number of β-blockers have been
introduced which claim to be more cardioselective, that is they
selectively block β_1-receptors. It is important to remember
that the selectivity of these β_1-blockers is not absolute and
larger doses will inhibit all β-receptors.

β-receptor antagonists are now used for the treatment of
angina pectoris, hypertension, cardiac arrhythmias, anxiety
attacks, thyrotoxicosis, migraine and glaucoma (ref.6). This
chapter will summarize the basic understanding of their
pharmacology emphasizing the properties of those drugs that are
reported to be cardioselective and those that have partial
agonist activity. In addition the recent work on their metabolism
and pharmacokinetics will be reviewed.

1.2   CLINICAL PHARMACOLOGY PROPERTIES

All of these drugs share the common property of
competitively blocking β-receptors, however they differ from one
another in relation to other pharmacological characteristics such
as cardioselectivity, partial agonist activity and membrane
stabilizing activity. Table 1.1 summarizes these other
characteristics for a number of the widely used β-blockers.

To understand the pharmacology of the β-receptor
antagonists it is important to remember the functions of the
receptor involved and the physiological or pathological
conditions under which they are activated. For example
β-blockade will have little effect on the normal heart but will
have significant effect when sympathetic control of the heart is
high as during exercise. The overall response to a β-blocker
may also be modified by other properties of the drug. Partial
agonist activity (see Table 1.1) is an important pharmacological
property of many of today's drugs, and will be discussed in more
detail later. Some of these drugs also have direct actions on
cell membranes, commonly described as membrane stabilizing, local
anesthetic and quinidine-like. The local anesthetic potency of
propranolol is approximately equal to that of lidocaine, while
that of oxprenolol is approximately half.
The quinidine-like effects may result in a general depression of
myocardial function and can cause death in large doses.

β-receptors can be further subdivided into β_1-receptors,
showing an equal affinity for norepinephrine and epinephrine,

TABLE 1.1   PHARMACOLOGICAL CHARACTERISTICS OF β-BLOCKERS

Drug	Partial Agonist Activity	Membrane Stabilizing Activity

1.1.1   <u>Non-Selective</u> $(\beta_1 + \beta_2)$
         <u>Blockers</u>

Drug	Partial Agonist Activity	Membrane Stabilizing Activity
Alprenolol	++	+
Bunolol	Ø	Ø
Nadolol	Ø	Ø
Oxprenolol	++	+
Penbutolol	Ø	+
Pindolol	++	$\pm$
Propranolol	Ø	++
Sotalol	Ø	Ø
Timolol	$\pm$	Ø

1.1.2   <u>Cardioselective</u> (β_1)
         <u>Blockers</u>

Drug	Partial Agonist Activity	Membrane Stabilizing Activity
Acebutolol	+	+
Atenolol	Ø	Ø
Metoprolol	Ø	$\pm$
Practolol	++	$\frac{+}{\emptyset}$
Tolamolol	Ø	$\pm$

Adapted from reference 6 with permission.

TABLE 1.2   SUBGROUPING OF β-RECEPTORS

β_1-Receptors	β_2-Receptors
Heart	Bronchial tissue
Aqueous humor producing	Peripheral blood vessels
parts of eye	Uterus
Renin secreting tissues	Glycogenolysis
of kidney	Glucagon
	Insulin secretion
	Tremor

and β_2-receptors, which have a greater affinity for
epinephrine (ref.8). In 1968 Dunlop and Shanks (ref.9) reported
on the discovery of the β-blocker, practolol, which appeared to
be cardioselective. Radioligand studies have also confirmed the
presence of two subsets of β-receptors (ref.10). The

subgrouping of β-receptors is shown in Table 1.2. Although
practolol is no longer used because it caused eye damage (ref.5)
several other cardioselective drugs have now become available,
notably metoprolol and atenolol. However cardioselectivity is a
relative phenomenon which appears to be dose dependent (ref.11).

Pringle et al. (ref.12) recently compared the
cardioselectivities of five β-receptor antagonists (acebutolol,
atenolol, betaxolol, metoprolol, and propranolol). Several
studies have demonstrated that acebutol (refs.13-15), atenolol
(refs.16,17), betaxolol (refs.17-19), and metoprolol (refs.15,
20-22) are more cardioselective than propranolol when the effects
on bronchial and peripheral β_2-vascular receptors are compared.
Pringle et al. (ref.12) selected doses of the drugs which were
expected to produce equivalent blockade of cardiac β_1-receptors.
The differential blockade of certain β_2-mediated isoproterenol
induced changes in physiological finger tremor (ref.23), forearm
blood flow (ref.14), diastolic blood pressure (ref.24) and
certain metabolic parameters (refs.25-27) can be used to
demonstrate the cardioselectivity of β-receptor antagonists.
Using this approach Pringle et al. (ref.12) found that
acebutolol, atenolol, betaxolol, and metoprolol are all more
selective at the β_1-receptor than propranolol and that atenolol
and betaxolol are more cardioselective than acebutolol.

It was hoped that the more cardioselective β-blockers would
be of benefit in asthmatic patients. Various workers
(refs.16,28) have shown that the β_1-selective blockers affect
resting respiratory function less and allow stimulation of β_2-
receptors by agonists during treatment. However, serious and
unpredictable reductions in ventilatory function can still occur
(ref.29).

Based on the distribution of β-receptors in the vasculature
it might be expected that β_1-selective blockers would be better
anti-hypertensive agents than non-selective drugs. During
epinephrine infusion, non-selective β-blockade results in an
unopposed α-receptor mediated vasoconstriction with a rise in
mean and diastolic blood pressure. Selective β_1-blockade would
not affect peripheral vascular function (ref.20). This has been
called vascular sparing. In practice, however, it is not
possible to demonstrate differences in efficacy with chronic
maintenance therapy with the different types of antagonist at

equipotent doses in hypertensive patients (refs.30,31).

β -blockade can also have an effect on carbohydrate balance. Catecholamines can cause hyperglycemia by stimulating glycolysis predominantly in the liver and skeletal muscle. In addition, a β_1-mediated mechanism may lead to an enhanced insulin response to a glucose load (ref.32). Non-selective β-antagonists might therefore produce undesirable effects in diabetes mellitus; these could be diminished by using β_1-selective drugs. Evidence so far does not suggest that prolonged use of β-antagonists results in any increased risk of patients developing diabetes (ref.33). In addition these drugs, whether selective or not, do not increase the risk of hypoglycemic episodes in insulin treated diabetes (ref.34), nor do they seem to disturb glucose control in a clinically important fashion in the majority of patients taking oral hypoglycemic agents (ref.35).

However the response to insulin induced hypoglycemia in diabetic patients may be influenced by β -blockade. Non-selective drugs without partial agonist activity impair the rate of recovery of blood glucose, whereas the effect of β_1-selective drugs is either undetectable or much reduced (refs.36,37). Non-selective agents block β_2-mediated gluconcogenesis in the liver (refs.38,39).

In summary cardioselective drugs do not appear to be more efficacious than non-selective β -antagonists. However, they may be preferable in patients with an asthmatic condition or in insulin-dependent disease. A strong case can be made that β -blockers of any type should not be used in these patients. This is particularly so today because of the wide variety of therapeutic agents available.

What is the picture regarding drugs with partial agonist activity? Theoretically β -blockers act as competitive antagonists preventing catecholamines interacting with β -receptors and it might be expected that they would have no pharmacological action of their own. In practice however some β -blockers also act as agonists when interacting with the receptor (ref.40). This partial agonist activity (PAA), also known as intrinsic sympathomimetic activity (ISA), is always less than full agonism and is combined with antagonistic activity. The amount of PAA may vary and it has been suggested that for certain drugs it may be selective (ref.41). Some of the

currently available drugs that possess this property include
pindolol, oxprenolol, alprenolol, and acebutolol.

β-Antagonists may slow resting heart rate by blocking the
small amount of sympathetic activity and allowing unopposed vagal
parasympathetic tone. In situations therefore where bradycardia
may be a problem drugs with PAA may be substituted as they affect
rising heart rate less (ref.42). It has also been suggested that
they may result in smaller reductions in cardiac output (ref.43)
and protect against cardiac decompensation (ref.44). However,
the data are conflicting with some studies showing that β-
blockers with PAA affect cardiac function less both at rest
(refs.44,46) and even on exercise (ref.46) whereas others have
demonstrated no significant difference (refs.47,48). However,
one of the problems making recommendations based on resting
parameters, where the effects of PAA may not be most pronounced,
is that such drugs act as competitive antagonists in the presence
of increased sympathetic activity. This may result in negative
inotropic effects with potentially serious clinical consequences,
indeed cardiac failure has been reported in patients taking
pindolol (ref.49).

β-Blockers with β_2-PAA may be advantageous in patients
with peripheral vascular disease. Experimental evidence on the
comparable effects of oxprenolol and propranolol on peripheral
blood flow in normal subjects is consistent with this proposal
(ref.50). However there is no clinical evidence that drugs with
PAA produce less peripheral vascular adverse effects in
compromised patients (ref.51).

In summary the evidence would suggest that β-blockers with
PAA are as effective as those without agonist activity in the
management of hypertension (ref.52) and angina (ref.53). However
it has also been reported that β-blockers with PAA may be less
effective in patients with effort and nocturnal angina pectoris
and in patients with severe ischemic heart disease (ref.54). A
recent supplement to the British Journal of Clinical Pharmacology
has focused on the clinical relevance of PAA (refs.41,52,53).

Before discussing the metabolism and pharmacokinetics of the
β-blockers I would like to briefly mention labetalol, which was
introduced in the United States in 1984, and the development of
ultra-short acting β-blockers. Labetalol has unique and complex
pharmacological properties; it exhibits both α, and β

non-selective β-adrenergic blocking activity (refs.55,56). It
also inhibits re-uptake of norepinephrine into nerve terminals.

The relative ability of labetalol to interact with α and
β-receptors differs among the four possible isomers (ref.57).
It also appears to possess PAA which is largely confined to β_2-
receptors (ref.58).

Ultra-short acting β-blockers may be advantageous in
treating critically ill patients, in the acutely ischemic patient
and in the pre-operative setting. Two such drugs have recently
been developed; the cardioselective drug, esmolol, and the non-
selective beta-blocker, flestolol. Each drug has a short half-
life and a short duration of action thus making it possible to
titrate the dose to the desired effect. The pharmacology and
pharmacokinetics of esmolol have been reported by several groups
(refs.59-61). Reynolds et al. (ref.59) reported that this ultra-
short acting β-blocker possesses minimal PAA or membrane
stabilizing activity and is β_1-selective. Its short duration of
action is due to rapid enzymatic hydrolysis by red blood cell
esterases, different from acetylcholinesterase and plasma
cholinesterase (ref.62). The short duration of action has three
major advantages in acute care situations: (1) a rapid titration
to a preferred steady-state level of β-blockade, (2) a rapid
adjustment to different steady-state levels of receptor blockade
if required by the changing status of the patient and (3) a rapid
disappearance of beta-blockade following discontinuation of the
infusion in the event of adverse effects. Similar advantages are
apparent with the non-selective agent, flestolol (ref.63).

1.3 THE PHARMACOKINETICS OF β-ANTAGONISTS

This section will concentrate on the more recent
pharmacokinetic data. In 1976 Johnson and Regardh (ref.64)
published a review of the clinical pharmacokinetics of the
β-blockers. Since that time new drugs have been introduced and
improved assay methods developed. These methods have led to a
better understanding of the protein binding, stereoselective
metabolism and polymorphic oxidation of these drugs. A recent
review by Riddell et al. (ref.65) updated the clinical
pharmacokintics of the β-blockers, including those recently
introduced.

All of the β-blockers are fairly rapidly absorbed from the

gastrointestinal tract. Hydrophilic drugs are generally less
well absorbed than lipophilic β-blockers. For example,
approximately only 50% dose of oral doses of atenolol and
nadolol are absorbed (refs.66,67). Following absorption,
lipophilic drugs including acebutolol, alprenolol, labetalol,
metoprolol, oxprenolol, and propranolol are subject to extensive
first pass metabolism. The magnitude of the effect depends on
the route and frequency of administration and may also be altered
by changes in hepatic blood flow and drug-metabolising activity
(ref.68). Concurrent food intake markedly reduces presystemic
clearance and increases systemic bioavailability (ref.69).

Generally β-antagonists are rapidly distributed from the
blood to the various tissues of the body and most have a larger
volume of distribution than total body volume, indicating binding
at extravascular sites (ref.65). The degree of plasma protein
binding varies from less than 5% (for atenolol and sotalol) to
more than 90% (for propranolol and oxprenolol). As basic drugs
they bind not only to albumin, but also to α_1-acid glycoprotein.
Generally the extent of protein binding increases with increasing
lipophilicity, but acebutolol, labetalol and metoprolol are
exceptions (ref.70).

Although some of the β-blockers, including atenolol,
nadolol and sotalol are excreted unchanged in the urine, the
majority are subject to highly variable first-pass metabolism
(ref.71). Labetalol (ref.72) is conjugated in its first-pass
metabolism, acebutolol is acetylated, although this is not under
genetic control (ref.73) and pindolol is conjugated and oxidized
(ref.71). Table 1.3 summarizes the major pharmacokinetic
parameters of the β-blockers.

It can be seen from Table 1.3 that the extent of metabolism
is highly variable and ranges from minimal to almost complete
metabolism. The extent of metabolism is related to the
lipophilicity of the β-blocker. Hydrophilic drugs such as
atenolol and nadolol are only slightly metabolised, whereas the
more lipophilic drugs, such as propranolol are virtually
completely metabolised. Bourne (ref.72) has pointed out the
excellent relationship between log P (log of the partition
between octanol and water) and the extent of metabolism of
several β-blockers in man. The routes of metabolism are
structure-dependent and are therefore, largely determined by the

TABLE 1.3

Drug	$t_{1/2}$ (h)	Vd (L/kg)	CL (L/h/kg)	Excreted unchanged	Metabolic pathway	Active metabolites	Significant first-pass metabolism	Oral Bioavailability	Protein Binding (%)
Acebutolol	7	1.2	0.29	app.15-20	Acetylation	Yes	Yes	20-60	app.20
Alprenolol	2.5	1-3.5	0.39	<1	Oxidation	Yes	Yes	app.10	76
Atenolol	6-7	1.1	0.16	100		No	No	50-60	<5
Betaxolol	16-22	6	0.28	16	Hydroxylation	No	No	80-89	50
Bevantolol	1.5	1.5		<10	Conjugation	No	No	60	app.30
Bisoprolol	10	3.2	0.22	48			No	90	
Bucindolol	2.5-8	app.100	6.5	<0.1	Oxidation		Yes	app.17	
Celiprolol	4-5		app.0.12	11-18			No	30-70	25
Esmolol	0.12-0.15	2-3.5	10-17		Hydrolysis	No			56
Flestolol	0.06-0.1	app.32	6-12		Hydrolysis	No			
Labetalol	3.9	5.6	1.3	<5	Conjugation		Yes	30-40	50
Metoprolol	3.5	5.5	0.97	<5	Oxidation	Yes	Yes	40-50	app.8
Nadolol	19	1.9	0.10	100		No	No	30-50	30
Oxprenolol	2.2	1.3	0.17	<5	Oxidation	No	Yes	25-60	92
Penbutolol	26			<10	Conjugation/ oxidation	?	No	app.100	>95
Pindolol	2.5-4	1.2-2	0.46	app.40	Conjugation/ oxidation		No	app.100	50-70
Practolol	10-11	1.6		100		No	No	95	
Propranolol	4	2.8-5.5	0.90	<1	Oxidation	Yes	Yes	app.30	90-94
Sotalol	7.5-15	1.3	0.14	75		No	No	app.100	21
Timolol	2.7	2.0-2.5	0.46	20	Oxidation	?	No	75	60

particular functional groups and the options that they confer.
Structurally the β-blockers are closely related and therefore are
metabolised by a narrow and common spectrum of metabolic
pathways.   Table 1.4 summarizes these pathways.

TABLE 1.4   PATHWAYS OF METABOLISM OF β-BLOCKERS

Metabolic Pathway	Examples
Aromatic Hydroxylation	Alprenolol, Oxprenol, Propranolol
O-Dealkylation	Metoprolol, Oxprenolol
N-Dealkylation	Propranolol
Oxypropanolamine Side Chain Dealkylation and Further Oxidation	Propranolol, Metoprolol
Glucuronic Acid Conjugation	Propranolol, Oxprenolol, Labetalol

The structures of these drugs afford opportunities for
C-oxidation and several β-blockers undergo the metabolic
reactions of aromatic hydroxylation and O- and N-dealkylation.
Following N-dealkylation the oxypropranolamine side chain is
subject to further oxidative metabolism leading to a glycol and
subsequently to lactic and acetic acid derivatives.   A further
metabolic pathway of major significance for disposition of
β-blockers is that of glucuronic acid conjugation.   This
frequently occurs at the secondary alcohol function on the side
chain and in this way the drug may be eliminated by direct
conjugation with glucuronic acid.   Alternatively the reaction
occurs following α-hydroxylation of the parent drug molecule.
Thus, both propranolol and oxprenolol are metabolised in man to
phenolic metabolites which are excreted as glucuronide
conjugates.
    A further complication in the metabolism of β-blockers is
that it may be stereoselective for those that are prescribed as
racemic mixtures.   The aliphatic hydroxyl group, which appears to
be essential for activity, also confers on the molecule optical
activity.   The levorotatory forms of both β-adrenergic agonists
and antagonists are much more potent than the dextrorotatory
forms.   This difference is useful in distinguishing the effects
of β-blockade from the other pharmacological effects of the
drugs.   For example the d isomer of propranolol has less than 1%

of the l isomer in blocking β -receptors, but the two are
equipotent as local anesthetics. When the racemic mixtures of β -
blockers are administered there is evidence that the disposition
of the enantiomers is different (ref.73). This is particularly
so for propranolol (ref.74) and metoprolol (ref.75).

Metabolic disposition may be clinically relevant for a
number of reasons including: (1) for those lipophilic drugs that
are extensively metabolised, metabolism is a key determinant of
their pharmacokinetics, (2) formation of active metabolites, (3)
stereospecific metabolism and (4) interindividual variation and
sources of variable metabolism.

The formation of active metabolites occurs with a number of
β -blockers. However, what has been difficult to assess is the
relative importance of these metabolites to the active
pharmacology of the drug. The formation of active metabolites
occurs with acebutolol, alprenolol, bopindolol, metoprolol, and
propranolol (Table 1.3) and may occur with other β -blockers.
Acebutolol for example is metabolised in part to an N-acetyl
derivative which is supposed to be as active as the parent drug
with respect to β -blocking properties (ref.76). The aromatic
hydroxylation of both alprenolol and propranolol, the major
metabolite of propranolol, to the overall β -blocking effect of
the drug has long been unclear. Recent evidence (refs.77-79)
suggests that its importance in this respect has been
overestimated.

There is extensive intersubject variation in the disposition
of a number of β -blockers and their pharmacological effects
(ref.65). For example the variation is about four-fold for
pindolol, seven-fold for metoprolol and ten to twenty-fold for
propranolol. By contrast, variation in the peak plasma level of
atenolol, which is essentially non-metabolised is low. For
propranolol a number of contributing factors have been identified
including age (ref.77), hepatic dysfunction, thyroid disease,
cigarette smoking (ref.77), and concomitant drug use. Even when
these factors are considered, considerable intersubject variation
still exists. It is now believed that the major cause of these
differences is a difference in hepatic metabolism, particularly
oxidative biotransformation. It is now apparent that the
oxidative metabolism of a number of lipophilic drugs is under
single gene control and that variable oxidation patterns arise

from the occurrence of allelomorphic variants of the gene in
population. One of these polymorphisms, the debrisoquine
polymorphism, is relevant to several β-blockers since their
oxidation appears to be regulated from the same locus as that
governing the allylic hydroxylation of debrisoquine.

The discovery of this polymorphism of drug oxidation arose
from observations on an affected individual who was unable to
metabolise debrisoquine normally. Subsequent population and
family studies showed that the trait of impaired drug oxidation
was a genetically determined recessive phenomenon transmitted in
an apparently simple Mendelian fashion (ref.78). Two phenotypes
were recognised, extensive metabolisers and poor metabolisers.
For further details the interested reader is referred to the
review by Idle and Smith (ref.79).

Several reviews (refs.80-82) have appeared on the
polymorphic metabolism of the β-blockers. The two drugs that
have been most extensively studied are metoprolol and
propranolol. Three studies (refs.83-85) have shown a strong
correlation between debrisoquine phenotype and the
pharmacokinetics of metoprolol. McGourty et al. (ref.85) studied
a large population (n=97) of hypertensive patients and found that
the mean plasma metoprolol concentration at 3 hours after oral
dosing (100 mg) was 3 times greater in poor metabolisers (mean $\pm$
SD, 262 $\pm$ 29 mcg/L) compared with extensive metabolisers (99 $\pm$ 53
mcg/L). They also reported that the plasma concentration
correlated significantly with the debrisoquine to
4'-hydroxydebrisoquine ratio. Differences in the metabolism of
the enantiomers of metoprolol have also been observed
(refs.84,86). In poor metabolisers the half-life of (R)-
metoprolol is longer than that of (S)-enantiomer whereas no
differences were observed in extensive metabolisers.

The α-hydroxylation of metoprolol and the 4'-hydroxylation
of debrisoquine both involve attack on a benzylic carbon. In
poor metabolisers the formation of α-hydroxymetoprolol was
impaired (ref.87); the metabolic clearance being almost 200 times
less in this group. The polymorphic nature of metoprolol
metabolism was confirmed by studies (refs.85,88) which showed a
bimodal distribution for the urinary ratio of metoprolol to
α-hydroxymetoprolol. Only approximately 10% of a dose of
metoprolol is recovered in the urine as the α-hydroxy metabolite

(ref.89), thus the large differences observed between poor and extensive metabolisers cannot be explained by a defect in this pathway. The most important route of metabolism is O-demethylation followed by the oxidation of the resulting primary alcohol. There was no clear separation into two phenotypes for this metabolic pathway (ref.85) suggesting that other factors contribute to the large differences in plasma metoprolol concentrations.

Do these pharmacokinetic differences have an effect on the clinical response to the drug? Regardh and Johnsson (ref.90) established a logarithmic relationship between plasma metoprolol concentration and its effect on heart rate. As expected, the β-blocking activity was found to be greater in poor metabolisers on single and multiple dosing (refs.83-85,91). The longer plasma half-life of metoprolol in poor metabolisers resulted in maintenance of β-blockade for 24 hours, not generally seen in extensive metabolisers even when slow-release preparations are used.

Early reports suggested that the pharmacokinetics and pharmacodymanics of propranolol may also be under genetic control (refs.74,92). However two later studies (refs.93,94) clearly showed that oral propranolol gives rise to both similar plasma concentrations and extent and duration of β-blockade in poor and extensive metabolisers. The formation of 4'-hydroxypropranolol is impaired in poor metabolisers, although this group still produces significant amounts of this metabolite. This together with the finding that antibodies raised to the debrisoquine P450 abolished virtually all debrisoquine 4'-hydroxylase activity, yet lowered the rate of 4'-hydroxypropranolol formation by only 60% (ref.95) implies that more than one enzyme is involved in the 4'-hydroxylation of propranolol at therapeutic doses. Although a significant percentage of propranolol is cleared from the body by oxidative reactions, the 4'-hydroxy metabolite accounts for only 20 to 40% of the dose (refs.93,96). The lack of any relationship between the pharmacokinetics of the parent drug and debrisoquine oxidation indicates that the other pathways are not under this type of genetic control. This has been confirmed for the formation of the glucuronide and glycol metabolites (refs.93,94). Polymorphic metabolism has also been demonstrated for two other β-blockers, bufural and timolol (refs.97,98).

There are several potential pharmacokinetic and clinical
consequences of polymorphic metabolism. Two are of interest to
the metabolism of β-blockers: (1) reduced first-pass metabolism
leading to increased plasma concentration and bioavailability
and (2) the possibility of competing substrates for the enzyme
system. The β-blockers have a wide therapeutic range and
therefore concentration dependent side effects are uncommon,
however in poor metabolisers a number of adverse effects have
been postulated to be a result of this genetic deficiency. Alvan
et al. (ref.99) have suggested that this group are likely to
develop unusually high concentrations of certain β-blockers and,
therefore, be at risk of concentration dependent side-effects
such as bradycardia and, for the β_1-selective drugs,
bronchoconstriction. Objective evidence for this has not been
presented.

Central nervous system related side-effects (ref.7) are more
commonly seen with the lipophilic β-blockers than with their
hydrophilic counterparts (ref.81). Whether or not the
development of these side-effects is concentration dependent is
unknown. If they are, then poor metabolisers would be at greater
risk than extensive metabolisers. The hydrophilic drugs, such as
atenolol, are said to cause fewer adverse effects on the central
nervous system than the lipophilic drugs. There is some
supporting evidence for this (refs.100-102).

Concurrent administration of a β-blocker with another drug
may cause changes in the pharmacokinetics of either or both
drugs. The potential for interactions is great because $-\beta$
blockers reduce liver blood flow. Some compete for binding to
α_1-acid glycoproteins and the lipophilic drugs compete for
hepatic metabolic pathways. Of these the competition for protein
binding sites is not clinically relevant (ref.103). The
pharmacokinetic drug interactions of propranolol have recently
been reviewed (ref.104). Probably one of the most important of
these interactions is that with cimetidine which reduces the
clearance of propranolol.

Plasma concentration monitoring of β-blockers is unlikely
to be helpful clinically for a number of reasons. For many of
these drugs the duration of the pharmacodynamic effect is much
longer than would be predicted on the basis of their
pharmacokinetic parameters. No meaningful range of effective

plasma concentrations has been established and even if free
concentrations are monitored there is little improvement in
obtaining a therapeutically useful range. Most of the drugs are
administered as racemic mixtures and there is evidence that the
disposition of the isomers is different (ref.105) ideally
therefore the concentration of the individual isomer should be
measured. However the vast number of analytical and
pharmacokinetic studies have resulted in much information which
is of theoretical and practical value. It is likely that as new
and more potent β-blockers are introduced, and techniques that
allow routine monitoring of the stereoisomers are developed, more
useful information will be obtained.

REFERENCES

1   R.P. Ahlquist, Am.J.Physiol., 153 (1948) 586-600.
2   C.E. Powell and I.H. Slater, J. Pharmacol. Exp. Therap., 122
    (1958) 480-488.
3   B.N.C. Prichard, C.J. Dickinson, G.A.O. Alleque, P. Hurst,
    J.D. Hill, M.L. Rosenheim, and D.R. Lawrence, Br. Med. J., ii
    (1963) 1226-27.
4   B.N.C. Prichard, Br. Med. J., 1 (1964) 1227-28.
5   C.D. Jackson and L. Fishbein, Fund. Appl. Toxicol., 6 (1986)
    395-422.
6   N. Weiner, in A.G. Gilman, L.S. Goodman, T.W. Rall and F.
    Murad (Editors), The Pharmacological Basis of Therapeutics,
    MacMillan, New York, 1985, pp. 181-214.
7   D.G. McDevitt, Drugs, 17 (1979) 267-288.
8   A.M. Lands, A. Arnold, J.P. McAuleff, F.P. Lunduena and T.G.
    Brown Jr., Nature, 214 (1967) 597-598.
9   D. Dunlop and R.G. Shanks, Br. J. Pharmacol., 32 (1968)
    201-218.
10 R.J. Lefkowitz, Life Sci., 18 (1975) 461-472.
11 J.J.L. Lertora, A.L. Mark, U.J. Johannsen, W.R. Wilson and
    F.M. Abound, J. Clin. Invest., 56 (1975) 719-724.
12 T.H. Pringle, J.G. Riddell and R.G. Shanks, J. Cardiovas.
    Pharmacol., 10 (1987) 228-237.
13 C.R. Kumana, C.M. Kaye, M. Leighton, P. Turner and J. Hamer,
    Lancet, ii (1975) 90-93.
14 G. Mougeot, F.C. Hughes, D. Julien and J. Marche, Arch. Int.
    Pharmacodyn., 251 (1981) 116-125.
15 M.S. Thomas and A.E. Tattersfield, Eur. J. Clin. Pharmacol.,
    29 (1986) 679-683.
16 M.E. Ellis, J.N. Sahay, S.S. Chatterjee, J.M. Cruickshank
    and S.H. Ellis, Eur. J. Clin. Pharmacol., 21 (1981) 173-176.
17 J. G. Riddell and R. G. Shanks, Clin. Pharmacol. Therap.,
    38 (1985) 554-559.
18 R. Palminteri and G. Kaik, Eur. J. Clin. Pharmacol., 24
    (1983) 741-745.
19 J. Saunders, R. Gomeni, J.R. Kilborn, P.L. Morselli and P.H.
    Sonksen, Eur. J. Clin. Pharmacol., 21 (1981) 177-184.

20 G. Johnsson, G. Nyberg and L. Solvell, Acta. Pharmacol.
    Toxicol., 36 (Suppl.5) (1975) 69-75.
21 G. Thiringer and N. Svedmyr, Eur. J. Clin. Pharmacol., 10
    (1976) 163-170.
22 W.R. Hiatt, D.C. Fradl, G.O. Zerbe, R.L. Byyny and A.S.
    Nies, Clin. Pharmacol. Therap., 35 (1984) 12-18.
23 J.M.O. Arnold and D.G. McDevitt, Br. J. Clin. Pharmacol.,
    18 (1983) 145-152.
24 J.M.O. Arnold and D.G. McDevitt, Br. J. Clin. Pharmacol.,
    16 (1983) 175-184.
25 J.M.O. Arnold, P.C. O'Conner, J.G. Riddell, D.W.G. Harron,
    R.G. Shanks and D.G. McDevitt, Br. J. Clin. Pharmacol., 19
    (1985) 619-630.
26 S.T. Holgate, W.A. Stubbs, P.J. Wood, E.S. McCaughey,
    K.G.M.M. Alberts and A.E. Tattersfield, Clin. Sci., 59 (1980)
    155-161.
27 H.H. Vincent, F. Boomsma, A.J. Man In't Veld, F.H.M. Derkx,
    G.J. Senting and M.A.D.H. Schalekamp, J. Cardiovasc.
    Pharmacol., 6 (1984) 107-114.
28 H. Formgren, Br. J. Clin. Pharmacol., 3 (1976) 1007-1014.
29 H.J. Waal-Manning and F.O. Simpson, Br. J. Clin. Pharmacol.,
    13 (Suppl.1) (1982) 655-735.
30 N. Clausen, T. Damsgaard and K. Mellemgaard, Br. J. Clin.
    Pharmacol., 7 (1979) 379-383.
31 A. Breckenridge, Br. Med. J., 286 (1983) 1085-1088.
32 A. Loubatieres, M.M. Mariami, G. Sorel and L. Savi,
    Diabetologia, 7 (1971) 127-132.
33 A. Vedin, C. Wilhelmsson and P. Bjorntorp, Acta. Med. Scand.,
    575 (Suppl.1) (1975) 37-40.
34 A.H. Barnett, D. Leslie and P.J. Watkins, Br. Med. J., 280
    (1980) 976-978.
35 A.D. Wright, S.G. Barber, M.J. Kendall and P.H. Poole, Br.
    Med. J., 1 (1979) 159-161.
36 M.M. Davidson, R.J.M. Corrau, T.R.D. Shan and E.B. French,
    Scott Med. J., 22 (1976) 69-72.
37 S.P. Deacon and D. Barnet, Br. Med. J., 2 (1976) 272-273.
38 A.J. Garber, P.E. Cryer, J.V. Santiago, M.W. Haymond, A.S.
    Paguari and D.M. Kipns, J. Clin. Invest., 58 (1976) 7-15.
39 I. Lager, G. Blohme and U. Smith, Lancet i (1979) 458-462.
40 J.W. Black, W.A.M. Duncan, R.G. Shanks, Br. J. Pharmacol.,
    25 (1965) 577-591.
41 W.H. Aellig and B.J. Clark, Br. J. Pharmacol., 24 (Suppl.1)
    (1987)
42 J.J. McNeil and W.J. Louis, Br. J. Pharmacol., 8 (1979) 1635
    -1665.
43 W.H. Aellig, Br. J. Clin. Pharmacol., 13 (1982) 1875-1925.
44 P. Imhof, Adv. Clin. Pharmacol., 11 (1976) 26-32.
45 T.L. Svendsen, O.J. Hartling, J. Trap-Jensen, A. McNair, and
    J. Bliddal, Clin. Pharmacol. Therap., 29 (1981) 711-718.
46 S.H. Taylor, B. Silke and P.S. Lee., New Engl. J. Med., 306
    (1982) 631-635.
47 J.A. Franciosa, S.M. Johnson and L.J. Tobian, Clin.
    Pharmacol. Therap., 26 (1979) 676-681.
48 B. Silke, S.P. Verma, R.C. Ahiya, M. Hussain, M. Hafizullah,
    G. Reynolds, G.I.C. Nelson, and S.H. Taylor, Eur. J. Clin.
    Pharmacol., 27 (1984) 509-515.
49 B. Davies, R. Bannister, C. Mathias, and P. Sever, Lancet, ii
    (1981) 982-983.
50 M.J. Vandenburg, C. Conlon and J.M Ledingham, Br. J. Clin.
    Pharmacol., 11 (1981) 485-490.

51 D.G. McDevitt, J. Cardiovas. Pharmacol., 8 (Suppl.6) (1986) 35-511.
52 A.H. Van Der Meiracker, A.J. Man In't Veld and M.A.D.H. Schalekamp, Br. J. Clin. Pharmacol., 24 (1987) 395-445.
53 P.A. Ades, Br. J. Clin. Pharmacol., 24 (Suppl.1) (1987) 295-345.
54 A.A. Quyyumi, C. Wright, L. Mockus and K.M. Fox, Br. Med. J., (1984) 951-953.
55 R.T. Brittain and G.P. Levy, Br. J. Clin. Pharmacol., 3 (1976) 681-694.
56 D.A. Richards and B.N.C. Prichard, Clin. Pharmacol. Therap., 23 (1978) 253-258.
57 E.J. Sybertz, C.S. Sabin, K.K. Pula, G.V. Vliet, J. Glennon, E.H. Gold and T. Baumm, J. Pharmacol. Exp. Therap., 218 (1981) 435-463.
58 T. Baum and E.J. Sylertz, Fed. Proc., 42 (1983) 176-181.
59 R.D. Reynolds, R.J. Gorczynski and C.Y. Quon, J. Clin. Pharmacol., 26 (1986) A3-A14.
60 R.J. Sung, L. Blanski, J. Kirshenbaum, P. MacCosbe, P. Turlapaty and A.R. Ladda, J. Clin. Pharmacol., 26 (Suppl.A) (1986) A15-A26.
61 V.S. Murthy, T.F. Hwang, B.W. Sandage and A.R. Ladda, J. Clin. Pharmacol., 26 (Suppl.A) (1986) A27-A35.
62 C.Y. Quon and H.S. Stampfli, Drug Metab. Dispos., 13 (1985) 420-424.
63 S.D. Barton, J. Burge, P. Turlapaty and A. R. Ladda, J. Clin. Pharmacol., 26 (Suppl.A) (1986) A36-A39.
64 G. Johnnson and C.R. Regardh, Clin. Pharmacokin., 1 (1976) 233-263.
65 J.G. Riddell, D.W.G. Harron and R.G. Shanks, Clin. Pharmacokin., 12 (1987) 305-320.
66 R.C. Heel, R.N. Brogden, G.E. Pakes, T.M. Speight and G.S. Avery, Drugs, 20 (1980) 1-23.
67 W.D. Mason, N. Winer, G. Kochak, I. Cohen and R. Bells, Clin. Pharmacol. Therap., 25 (1979) 408-415.
68 C.F. George, Clin. Pharmacokin., 8 (1983) 286-293.
69 A. Melander and A. McLean, Clin. Pharmacokin., 8 (1983) 286 -293.
70 D.B. Jack, Br. J. Clin. Pharmacol., 11 (1981) 402-406.
71 A.J. Smith and G.T. Tucker, Kinetics and Biotransformation of Adrenergic Inhibitors: Adrenergic Activators and Inhibition, In Handbook of Experimental Pharmacology, Springer-Verlag, Berlin, 1980, pp. 417-509.
72 G.R. Boume, The Metabolism of β -adrenoceptor blocking drugs, In J.W. Bridges and L. Chasseaud (Editors), Progress in Drug Metabolism, Vol.6, Wiley, London, pp. 77-110.
73 K. Williams and E. Lee, Drugs, 30 (1985) 333-356.
74 C. Von Bahr, J. Hermansson, and K. Tawara, Br. J. Clin. Pharmacol., 14 (1982) 79-82.
75 M.S. Lennard, G.T. Tucker, J. H. Silas, S. Freestone, L.E. Ramsey and H.F. Woods, Clin. Pharmacol. Therap., 34 (1983) 732-737.
76 C.M, Kaye, C.R. Kumana, M. Leighton, J. Hamer and P. Turner, Clin. Pharmacol. Therap., 19 (1976) 416-420.
77 R.E. Vestal, A.J.J. Wood, R.A. Branch, D.G. Shand and G.R. Wilkinson, Clin. Pharmacol. Therap., 26 (1979) 8-15.
78 A. Mahgoub, J. R. Idle, L.G. Dring, R. Lancaster and R.L. Smith, Lancet, 2 (1977) 584-586.

79   J.R. Idle and R.L. Smith, The debrisoquine hydroxylation
     gene: a gene of multiple consequences, In L. Lemberger
     and M.M. Reidenberg (Editors), Proceedings of the Second
     World Congress of Clinical Pharmacology and Therapeutics,
     Washington D.C., 1984, pp. 148-164.
80   J.H. Silas, M.S. Lennard, G.T. Tucker, L.E. Ramsay and H.F.
     Woods, Br. J. Clin. Pharmacol., 17 (1984) 115-185.
81   R.L. Smith, Eur. J. Clin. Pharmacol., 28 (Suppl.) (1985)
     77-84.
82   M.S. Lennard, G.T. Tucker and H.F. Woods, Clin. Pharmacokin.,
     11 (1986) 1-17.
83   M.S. Lennard, J.H. Silas, S. Freestone, L.E. Ramsay, G.T.
     Tucker and H.F. Woods, New. Engl. J. Med., 307 (1982)
     1558-1560.
84   P. Dayer, R. Gasser, J. Gut, T. Kronback, G.M. Robertz,
     et al., Biochem. Biophys. Res. Comm., 30 (1984) 374-380.
85   J.C. McGourty, J.H. Silas, M.S. Lennard, G.T. Tucker and
     H.F. Woods, Br. J. Clin. Pharmacol., 20 (1985) 555-566.
86   M.S. Lennard, G.T. Tucker, J.H. Silas, S. Freestone, L.E.
     Ramsay and H.F. Woods, Clin. Pharmacol. Therap., 34 (1983)
     732-737.
87   M.S. Lennard, G.T. Tucker, J.H. Silas and H.F.Wood,
     Xenobiotica.
88   M.S. Lennard, S. Freestone, L.E. Ramsay, G.T. Tucker and H.F.
     Woods, New Engl. J. Med., 308 (1983) 965-966.
89   K.O. Borg, E. Carlsson, K.J. Hoffmann, T.E. Johnsson, H.
     Thorin and B. Wallin, Acta. Pharmacol. Toxicol., 36 (1975)
     125-135.
90   C.G. Regardh, Clin. Pharmacokin., 5 (1980) 557-569.
91   S. Freestone, J.H. Silas, M.S. Lennard and L.E. Ramsay, Br.
     J. Clin. Pharmacol., 14 (1982) 713-718.
92   R.R. Shah, N.S. Oates, J.R. Idle and R.L. Smith, Lancet, 1
     (1982) 508-509.
93   M.S. Lennard, P.R. Jackson, S. Freestone, G.T. Tucker, L.E.
     Ramsay and H.F. Woods, Br. J. Clin. Pharmacol., 17 (1984)
     679-686.
94   T.C. Raghuram, R.P. Koshakji, G.R. Wilkinson and A.J.J. Wood,
     Clin. Pharmacol. Therap., 36 (1984) 51-56.
95   L.M. Distlerath and F.P. Guengerich,. Proc. Natl. Acad. Sci.,
     81 (1986) 7348-7352.
96   T. Walle, U.K. Walle and L.S. Olanoff, Drug Metab. Disp., 13
     (1985) 204-209.
97   P. Dayer, A. Kuble, A. Kupfer, F. Courvoisier, L. Balant and
     J. Fabre, Br. J. Clin. Pharmacol., 13 (1982) 750-752.
98   R.V. Lewis, M.S. Lennard, P.R. Jackson, G.T. Tucker, L.E.
     Ramsay and H.F. Woods, Br. J. Clin. Pharmacol., 18 (1984)
     287P.
99   G. Alvan, C. von Bahr, P. Seideman and F. Sjoquist, Lancet,
     1 (1982) 333.
100 O. Lynstam and L. Ryden, Acta. Medica. Scand., 209 (1981)
     261-266.
101 J.R. Cove-Smith and C.A. Kirk, Eur. J. Clin. Pharmacol., 28
     (Suppl.) (1985) 21-23.
102 J.B. Kostis and R.C. Rosen, Clin. Pharmacol. Therap., 39
     (1986) 203.
103 C.G. Regardh, Acta. Medica. Scand., 212 (Suppl.665) (1982)
     49-60.
104 A.J.J. Wood and J. Feely, Clin. Pharmacokin., 8 (1983) 253
     -262.
105 T. Walle, J.G. Webb, E.E. Bagwell, U.K. Walle, H.B. Daniell
     and T.E. Gaffney, Biochem. Pharmacol., 37 (1988) 115-124.

Chapter 2

CLINICAL PHARMACOKINETICS OF BETA-BLOCKERS

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2.1 INTRODUCTION

Beta-adrenoceptor antagonists were introduced into clinical practice more than 20 years ago. Since then a huge amount of data concerning their pharmacokinetics and metabolism has accumulated. There is hardly another class of drugs whose clinical pharmacokinetics have been studied as thoroughly as those of beta-blockers. It seems impossible to review all published data on the kinetics and metabolism of beta-adrenoceptor antagonists and also unnecessary, as many review articles dealing with the kinetics and biotransformation of beta-blockers, or of a certain member of this group of drugs, have appeared in the course of the last two decades (refs. 1-22). It is generally accepted that the chemist working on the analysis of beta-blockers in biological material should have a working knowledge of their clinical pharmacokinetics. The aim of this chapter is to review those partial problems of the clinical pharmacokinetics of beta-blockers which the author considered /1/ to be most relevant for analytical chemists working in the field and /2/ not to be covered extensively enough by the aforementioned literature. It is obvious that the selection of the topics to be discussed was highly subjective. With respect to the limited size of the chapter, those topics which were considered already to have been thoroughly reviewed have either been completely omitted or are discussed only briefly. On the other hand, some problems which hitherto have not been treated in detail have been given particular attention.

2.2 BASIC INFORMATION ON PHYSICO-CHEMICAL PROPERTIES AND PHARMA-COKINETICS OF BETA-BLOCKERS

The basic physico-chemical properties of beta-blockers are shown in Table 3.1. It can be seen that all clinically used beta-blockers are bases and their pK_a values are within the range 9.2--9.8. All contain an optically active carbon in their molecule. They are used in clinical practice as racemic mixtures, except penbutolol and timolol, for which the pharmacologically active l-enantiomer is available in a sufficient amount. Further, it can be seen that there are great differences in the lipid solubilities of the beta-blockers listed in Table 3.1. A significant structure-pharmacokinetic relationship has been established for beta-adreno-ceptor antagonists. Most of the studied pharmacokinetic parameters depend on the apparent octanol/buffer (pH 7.4) partition coeffi-cient. Significant correlations also exist among the pharmaco-kinetic parameters tested. Lipophilicity/hydrophilicity was the major determinant controlling the pharmacokinetic behaviour of all compounds studied. In particular, a log-log linear relationship was found to exist between the true octanol/water partition co-efficient and the following pharmacokinetic parameters: partition coefficient of the drug between plasma protein and plasma water, ratio of the fraction of the drug bound and unbound to albumin, true red cell partition coefficient, steady-state distribution volume relative to the unbound drug in plasma, ratio of the frac-tion of the drug bound and unbound in tissue and ratio of the fractions of the drug eliminated non-renally and renally (ref. 23).

The rate and extent of absorption of almost all clinically used beta-blockers from the gastrointestinal tract are high; a lesser extent of absorption is observed with hydrophilic beta-blo-ckers.

2.3 BLOOD LEVELS OF BETA-BLOCKERS

The biological material to be analysed for beta-blockers in order to monitor therapeutic levels or for research in clinical pharmacokinetics will probably be blood and more specifically one of its main components, plasma or serum. Generally, the plasma or serum concentrations of beta-blockers administered in therapeutic doses vary over a very wide range. Great differences are seen between the peak and the lowest still pharmacodynamically effec-tive levels of a given beta-blocker administered in a therapeutic
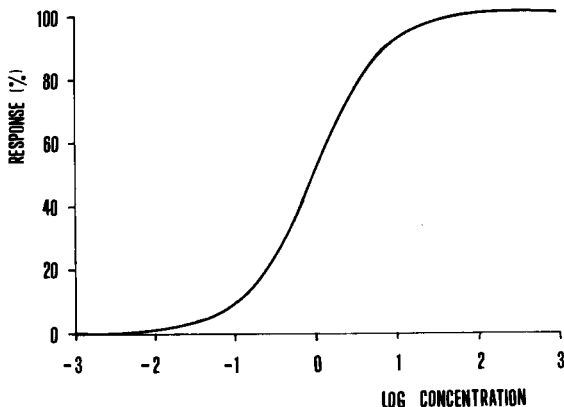
Fig. 2.1. Concentration-response curve (equation 2.4) showing a nearly linear dependence between 20% and 80% of the maximal response.

30), carteolol (ref. 31), alprenolol (refs. 32, 33), pindolol (refs. 34-36), propranolol (refs. 26, 37-42), atenolol (refs. 36, 43-45), tolamol (ref. 46), sotalol (ref. 47), pamatolol (ref. 48), metoprolol (ref. 40), practolol (ref. 49), esmolol (ref. 50), befunolol (ref. 41), bufuralol (ref. 51), timolol (refs. 39, 52) and nadolol (ref. 36).

To approximate the time course of the pharmacodynamic effect equation 2.1 was combined with the relationship describing the time course of the drug concentration in plasma after its administration under in vivo conditions and solved for R vs. t (ref. 53). The simplest pharmacokinetic situation is reflected by a one-compartment open linear model in which the plasma concentration of the drug after an intravenous bolus input decreases monoexponentially with time:

$$C = C_o \, e^{-kt} \tag{2.2}$$

where C_o is concentration at $t = 0$ and k is the elimination rate constant. Combination of equations 2.1 and 2.2 (for details see ref. 54) gives

$$R = R_o - (k \ m \ t)/2.3 \qquad\qquad (2.3)$$

where R_o is the intensity of the pharmacological response at $t = 0$ and m is the slope of the log(concentration)-response curve (equation 2.1). It is obvious from equation 2.3 that for the models used (equations 2.1 and 2.2) the pharmacological response decreases linearly with time. Equation 2.3 has been repeatedly used satisfactorily for the description of the time course of the pharmacological effect of beta-blockers (atenolol (ref. 45), propranolol (ref. 55), metoprolol (ref. 56)) and its validity has been generally accepted (ref. 57).

Propranolol levels associated with a given degree of blockade of exercise-induced tachycardia were found to be several times greater after intravenous administration than after oral administration. An active metabolite found only after the drug is taken by mouth (refs. 37, 58) was suggested to be involved.

Some beta-blockers proved to be antihypertensive agents. However, the relationship between the blood level of a drug with beta-blocking potency and the reduction of blood pressure is not as straightforward as in the case of tachycardia reduction. Thus the hypotensive effect was not correlated with the blood level of atenolol (ref. 44), propranolol (refs. 24, 59), penbutolol (ref. 27) and metoprolol (refs. 60, 61). On the other hand, a correlation between the reduction of isoproterenol- (for esmolol (ref. 50)) or exercise-induced (for acebutolol (ref. 62)) increases in systolic blood pressure and the logarithm of the drug concentration was demonstrated. A relationship between the peak concentration of pindolol in plasma and the maximal change in blood pressure in 15 previously untreated hypertensive patients was also reported (ref. 63). The pharmacokinetic differences have been suggested to be the main source of variation in blood pressure response to some beta-blockers (ref. 63).

The clinical effect of most beta-adrenoceptor blockers lasts much longer than would be expected on the basis of their elimination half-lives. Bopindolol may serve as an example. It has beta-blocking effects for 96 h despite a 4.5 h elimination half-life (ref. 64). To explain this apparent temporal dissociation between the pharmacokinetics and pharmacodynamics of beta-blockers, deep compartments and active metabolites were sought or "tight" receptor binding was suggested.

The approach that has evidently satisfied many so far un-

answered questions concerning the pharmacodynamic-pharmacokinetic relationship for beta-blockers will be outlined in the following section.

On introducing equation 2.1 we emphasized its empirical nature and its validity within the range of 20-80% of the maximum response (see Fig. 2.1). It follows that a linear decline of effects (equation 2.3) after a single dose of beta blocker obviously accounts for the observation only in a restricted range of effects (20-80% of the maximum) and fails completely if one tries to interpret the long lasting effects of drugs with short elimination half-lives from plasma (ref. 65).

A better description of the response vs. concentration data, shown schematically in Fig. 2.1, is given by the Hill equation:

$$R = R_m \; C^s/(1/Q + C^s) \tag{2.4}$$

where R is the intensity of pharmacological response corresponding to drug concentration C, C is the concentration of drug at time t which elicits the response R, R_m is the maximum intensity of the pharmacological response, s is the slope of a plot of $\ln(R/(R_m - R))$ vs. ln C and Q is the value of $R/(R_m - R)$ when C = 1. A combination of equation 2.4 with equation 2.2 (analogous to the combination of equations 2.1 and 2.2) and solution as R vs. t (for details see ref. 66) gives

$$R = (R_m \; C_0^s \; e^{-k \; s \; t})/(1/Q + C_0^s \; e^{-k \; s \; t}) \tag{2.5}$$

A cartesian plot of equation 2.5 (Fig. 2.2) shows that the response is essentially a linear function of time only within the 20-80% response range. Beta-adrenoceptor antagonists interact in a competitive manner with endogenous antagonists at the receptor site and their concentration-effect relationship should obey the law of mass action shown as the general log-linear function in Fig. 2.1.

In addition to progress in modelling, the results of studies on the binding of beta-adrenoceptor blocking drugs to beta adrenergic receptors has contributed considerably to our understanding of the mechanism of the long-lasting effects of some beta-blockers. A linear relationship between in vivo inhibition of exercise-induced tachycardia and in vitro inhibition of beta-adrenoceptor binding of the antagonist radioligand has been established for
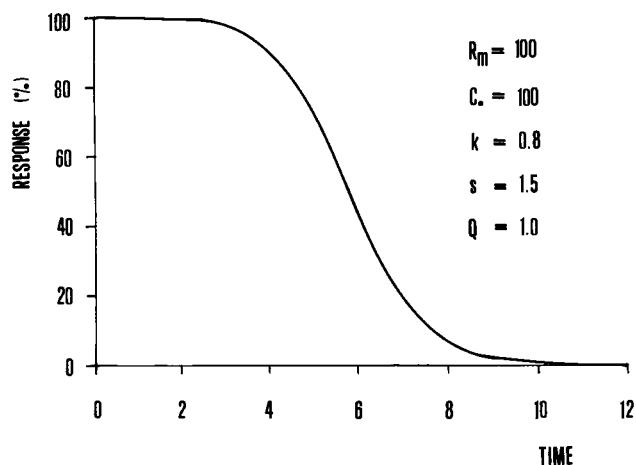
Fig. 2.2. Time course of response (equation 2.5) showing a linear relationship in the 20-80% response range.

several beta-blockers. This shows that plasma concentrations inducing antagonistic effects in vitro are representative of concentrations in the effect compartment for human beta-adrenoceptors. Further, for propranolol the EC_{50} values in vivo (for inhibition of exercise tachycardia, 8 ng/ml; range 3-15 ng/ml) and in vitro (competition of antagonist radioligand and ($\pm$)-propranolol at rat reticulocyte membrane adrenoceptors, 8-12 ng/ml) are in agreement (ref. 67). The same has also been found for penbutolol and atenolol (refs. 68, 69). It can be seen from the C vs. t plot for propranolol in human volunteers that the plasma concentrations close to the half-maximal effective concentration obtained from the in vitro receptor binding studies (8 ng/ml) were achieved between 18 and 22 h after administration. From the R vs. t plot (equation derived analogously to equation 2.5, except that instead of equation 2.2 an equation describing C vs. t data for oral drug administration was used), it follows that the time interval for the initial effect of propranolol to decline by half is 4- 5 times longer than the elimination half-life from plasma, i.e., 20.6 h, and a loss of effect ($<$10%) is predicted on average for 30 h (ref. 65). Fig. 2.3, reproduced from the work by Wellstein (ref. 65), depicts the relationship between drug concentration, effect and elimination half-life; assuming that the dose of the drug (e.g.
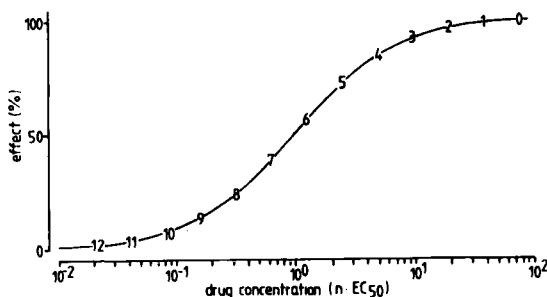
Fig. 2.3. Relationship between drug concentration, effect and eli-
mination half-life. The half-lives are denoted by the numbers
within the curve. Reproduced from A. Wellstein et al. Arzneim.-
-Forsch., 35 (1985) 2-6, with permission of the copyright owner.

beta-blocker) given to a patient is sufficient to achieve a plasma
concentration 80 times the respective EC_{50}, this amount will de-
cline by half every elimination half-life and will thus reach the
EC_{50} value after 6-7 elimination half-lives (the half-lives are
denoted by the numbers within the curve).

The data on bopindolol (ref. 64) are essentially in accord
with the above conclusions. The concentration of bopindolol in
plasma (predicted with a one-compartment body model) could be
related to the measured effects by classical effect models for 20
half-lives. The minimal effective plasma concentration of 1 pmol/l
suggests that a sufficient number of receptors is occupied at
chemically unmeasurable levels in plasma to induce the effect.

2.5 ELIMINATION KINETICS OF BETA-ADRENOCEPTOR BLOCKING DRUGS

Recognition of non-linearity in the kinetics of a drug can be
of great importance for its use in practical pharmacotherapy.
Wagner (ref. 70) defined kinetic linearity as direct proportio-
nality of transfer rates to concentrations or concentration diffe-
rences. This can be expressed by the relationship

$$dC/dt = k \, C \tag{2.6}$$

where C denotes the concentration of the drug, k the rate constant and t time. It follows that peak plasma concentration (C_{max}) vs. dose, area under the concentration of the drug in plasma (AUC) vs. dose and concentration of the drug under steady-state conditions (C_{ss}) vs. dose rate (Fig. 2.4) are linear relationships.
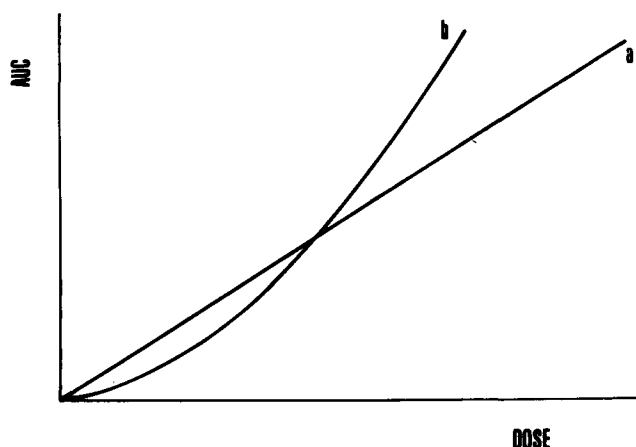

Fig. 2.4. Relationship between the administered dose and area under the plasma concentration of the drug in linear (a) and non--linear (b) pharmacokinetics.

    With non-linear kinetics the above relationships are non-linear with respect to the dose of the drug. The most common type of non-linear kinetics, the Michaelis-Menten elimination kinetics, can be expressed by the relationship

$$dC/dt = V_{max} \ C/(K_m + C) \tag{2.7}$$

where V_{max} is the maximal velocity and K_m is the Michaelis constant. For Michaelis-Menten kinetics the dependences of C_{max} and AUC on the dose have the form of concave curves (Fig. 2.4). Increasing doses of the drug will thus be accompanied by a disproportionate increase in its levels in blood, with pharmacodynamic and toxicological consequences.

    Clinically used beta-adrenolytics have been investigated with respect to linearity of their kinetics. Almost all have linear

pharmacokinetics: oxprenolol (refs. 71-73), metipranolol (ref. 74), atenolol (refs. 43, 44, 75), pamatolol (ref. 48), pindolol (refs. 76-78), medroxalol (ref. 79), cetamolol (ref. 75), nadolol (ref. 80), bevantolol (ref. 81), indenolol (ref. 82), acebutolol (ref. 83), metoprolol (ref. 84), timolol (refs. 52,85) and esmolol (ref. 86).

For celiprolol linear kinetics were reported in young volunteers (ref. 87), whereas in elderly volunteers a non-linear increase of AUC with increasing dose was observed (ref. 88). Non-linearity was also reported in sotalol pharmacokinetics, as after 25, 50 and 100 mg doses of the drug the mean plasma concentration was 1.36 μg/ml per 100 mg and after 200, 400 and 800 mg it was 0.57-0.69 μg/ml per 100 mg (ref. 47).

The elimination kinetics of propranolol, the most frequently prescribed beta-adrenolytic drug, have attracted much attention in the last two decades. The apparently controversial issues concerning the elimination kinetics of propranolol in man were mostly due to:

/1/ wide differences in doses of propranolol and in their range in various studies,

/2/ insufficient specificity or sensitivity of the assay methods for propranolol and

/3/ great inter- and intra-individual variability in the elimination kinetics of propranolol.

On the basis of a large number of pharmacokinetic studies it seems to be well established that the elimination kinetics of propranolol in man are dose dependent (refs. 89-93). This manifests itself as a disproportionate increase in the propranolol plasma level, in terms of AUC or C_{max}, with increasing propranolol dose. Thus, as oral doses of propranolol were increased from 20 to 320 mg, there was a decrease in intrinsic plasma clearance from 425 to 200 l/h and the half-life rose from 3 to 5 h (ref. 94). Silber (ref. 92) observed a 56% reduction in the intrinsic clearance and a 175% increase in the half-life of propranolol over the range of doses administered (40-320 mg/day). Concerning the mechanism of the non-linearity observed he concluded that the saturable elimination of propranolol could be explained only partly by saturability in the metabolic pathways resulting in the formation of propranolol glucuronide, 4-hydroxypropranolol glucuronide and α-naphthoxylactic acid. Wagner (ref. 95) has shown that the equation

$$C_{ss} = (K_m \quad R_o)/(V_m - R_o) \tag{2.8}$$

where C_{ss} is the average steady-state concentration when R_o is continued to the steady state, K_m is the pooled Michaelis constant, R_o is the zero-order input rate and V_m is the pooled maximal velocity of metabolism, is applicable to the Silber's data (ref. 92). He showed that the pooled Michaelis-Menten parameters V_m and K_m adequately describe the propranolol C_{ss} vs. R_o data and that the bioavailability of first-pass drugs that obey Michaelis-Menten kinetics, as demonstrated on propranolol, will be sensitive to the rate of drug input. In parallel, McAinsh (ref. 96), in a theoretical study demonstrated the importance of the magnitude of the absorption rate constant in the calculation of the area under the curve for drugs eliminated by a saturable elimination process. This may explain the lower bioavailability of propranolol after a sustained release formulation and also the decreased bio-availability of propranolol when the number of subdivisions of the daily dose is increased (ref. 95).

Concluding this section, we should mention the data of Walle (ref. 97), who has shown that an oral dose of propranolol can be used under rigorously controlled conditions, to predict a narrow plasma concentration range during chronic propranolol therapy within the therapeutic dosage range of 40-960 mg daily. This is in contrast with the results of a more recent study (ref. 98) in which the inter-individual variability was so large that this relationship had no predictive value. On the other hand, the intra-patient dose-concentration relationship was strong.

2.6 CHRONOPHARMACOKINETICS OF BETA-BLOCKERS

The time dependence of the pharmacokinetics of drugs is a well established fact. As the effectiveness of a drug also depends on its pharmacokinetic behaviour, possible rhythmic variation along the 24-h scale in drug distribution, metabolism and excretion may contribute to the chronopharmacodynamics of a drug (ref. 99). Data on the chronopharmacokinetics of beta-blockers in man are scarce and the predominant part of the information on this topic is based on experiments in animals. The data that were available can be summarized as follows: The half-life of nadolol, an unmetabolized beta-blocker, was longer at night than during the day, probably because of the slower nocturnal flow of urine (ref.

80). In another study, a single oral dose of propranolol was admi-
nistered to synchronized volunteers at each of the four different
test times: 8 a.m., 2 p.m., 8 p.m. and 2 a.m. Both C_{max} and AUC
were lowest after propranolol was administered at 2 p.m. (ref.
100). In rats the half-lives of propranolol, metoprolol, sotalol
and atenolol in plasma and organs were shorter during dark than
during light periods. No obvious temporal dependence was found in
other pharmacokinetic parameters (AUC, Cl, $V_{d\ beta}$), with the
exception of $V_{d\ beta}$ of propranolol (refs. 101-103).

2.7 ALTERATIONS IN THE PHARMACOKINETICS OF BETA-BLOCKERS DURING CHRONIC REGIMENS

In most indications beta-blockers are administered repeatedly
over long periods of time. Under chronic regimens the plasma con-
centrations may increase because the drug accumulates or inhibits
its own metabolism, or the concentrations may decrease if the
agent is an enzyme inducer. The changes in the pharmacokinetics of
beta- blockers appearing during long-term administration may lead
to serious pharmacodynamic consequences. Pharmacokinetic analysis
of chronic administration should therefore be obligatory for each
clinically used beta-adrenoceptor blocking drug. Before surveying
data on the pharmacokinetics of repeatedly administered beta-blo-
ckers, it is necessary to recall that the elimination half-life of
the drug determines the rate at which a certain degree of the
steady- state plasma concentration is attained and clearance de-
termines the plasma concentration of the drug at the steady state.
This can readily be seen from the following relationships (ref.
104):

$$C/C_{ss} = 1 - (1/2)^n \qquad (2.9)$$

and

$$C_{ss} = (\text{rate of administration})/(\text{clearance}) \qquad (2.10)$$

where C is plasma concentration, C_{ss} is plasma concentration at
the steady state and n is time in multiples of the half-life (n =
$t/t_{1/2}$). Both half-life and clearance are pharmacokinetic para-
meters that can be obtained from a single dose study. The plasma
level measured during chronic dosing can be compared with that
calculated from single administration parameters. Beta-blockers

are almost exclusively metabolized by the liver. The hepatic clea-
rance is

$$Cl_h = Q \quad E \qquad\qquad\qquad (2.11)$$

where Q is the liver blood flow and E is the hepatic extraction
ratio of the drug (ref. 54). It was shown that beta-blockers dimi-
nish the liver blood flow, which consequently decreases the hepa-
tic clearance of drugs (ref. 105) as seen from the above relation-
ship.

For several beta-blockers it has been reported that their
pharmacokinetic parameters remain constant during chronic admi-
nistration; no differences were observed between oxprenolol mean
plasma profiles after a single dose of oxprenolol given on day 1
and day 8 (ref. 106). For sotalol there was no induction of meta-
bolism or increase in efficiency of excretion during long-term
administration of sotalol (ref. 107). It was reported for betaxo-
lol that its pharmacokinetic characteristics were unchanged after
repeated doses (ref. 108) and for mepindolol that no appreciable
accumulation of the drug and/or active metabolites occurred during
the study period (ref. 109). The plasma elimination half-life,
area under the plasma concentration-time curve and peak plasma
concentration time after the administration of 200 mg of atenolol
were not changed by chronic dosing for 8 days (ref. 43). The AUCs
for timolol were similar following the first and the fourth doses
(ref. 52). Pindolol was given daily for 4 weeks and the kinetic
parameters remained stable (ref. 110). In another study the
steady-state concentration of pindolol was predicted from the
pharmacokinetic data obtained after a single dose (ref. 76). The
pharmacokinetic parameters of pindolol were the same for Rhesus
monkeys that received pindolol for the first time as for those
animals that had undergone a 5-year chronic treatment (ref. 111).
For indenolol no accumulation was reported in plasma after re-
peated administration (ref. 82). The blood clearance of acebutolol
was found to remain essentially constant over approximately 3
weeks (ref. 83). For timolol the elimination rate constant was
essentially unchanged after the tenth tablet compared with the
first (ref. 85). After treatment for 30 days with penbutolol at 40
mg/d there was no accumulation of the parent drug (ref. 112).

In other studies alterations of the kinetic parameters were
reported. For propranolol, after administration of an 80 mg single

dose the peak plasma level was 50 ng/ml and the elimination half-life 2.6 h. After chronic dosing for 14-21 days at 80 mg/day the peak level and the elimination half-life were approximately doubled (ref. 38). For an explanation of these changes removal into a large extravascular distribution volume and not actual clearance from the body by either excretion or metabolism was suggested. Similarly, in another study (ref. 41) the elimination half-life of propranolol on repeated administration (6.56 h) was found to be significantly prolonged in comparison with a single dose (1.86 h). On chronic dosing, an increase in the AUC was observed for propranolol, probably due to a progressive increase in the oral bioavailability of the drug (ref. 113). Similarly, both oral and systemic clearances of propranolol were lower after the seventh dose than after a single dose when 80 mg of propranolol in conventional form were taken three times a day (ref. 114). In line with these data, after administration of regular propranolol, the mean unbound oral clearance at the steady state decreased by 29% compared with a single dose. Similarly the oral clearance decreased by 33% with sustained-release propranolol. The accumulation ratios were 1.39 and 1.61, respectively. The percentage of unbound drug did not differ between single and multiple doses. The data support a decrease in the unbound intrinsic clearance of propranolol with no change in the unbound fraction, leading to an increase in bioavailability at steady state (ref. 115). For metoprolol the AUC and C_{max} increased significantly during repeated administration of the drug (refs. 116-119). Multiple doses of timolol led to an increase in the elimination half-life and bioavailability and to decrease in total clearance and distribution volume (ref. 39). For metipranolol a half-life of 4 h after a single dose compared with 2.5 h after chronic oral administration was reported (ref. 120). For celiprolol, in addition to an increase in the plasma elimination half-life, little change in the major pharmacokinetic parameters was reported between days 1 and 7 during repeated dose therapy (ref. 88).

2.8 POLYMORPHIC OXIDATION OF BETA-BLOCKERS

The large inter-individual variability of the pharmacokinetics and pharmacodynamics of some beta-blockers has been known since their introduction into clinical practice. Many of the lipophilic beta- blockers after being absorbed from the gut are eliminated metabolically in a highly variable manner during their

first pass through the liver. In the clearance of beta-blockers by
biotransformation the oxidation reactions play an important role.
Genetic polymorphism of oxidative metabolism has been recognized
as one of the sources contributing to the above-mentioned inter-
individual variability (for details see the review by Lennard
(ref. 121)). It has been shown that the oxidative metabolism of
some beta-blockers, similarly to that of some other drugs, is
regulated genetically and is linked to the debrisoquine oxidation
phenotype (refs. 122-124). The hydroxylation of debrisoquine (an
antihypertensive agent) in man exhibits genetic polymorphism, with
about 10% of the Caucasians being poor metabolizer phenotype and
the remaining 90% extensive metabolizer phenotype (refs. 125,
126). The test by which the subjects can be allocated to the res-
pective phenotype consists of measuring the ratio of debrisoquine
to 4-hydroxydebrisoquine in an 8-hour urine collection after a 10
mg oral dose of the drug. The distribution of this ratio is bimo-
dal in Caucasians. 4-Hydroxylation of debrisoquine is controlled
by two alleles at a single gene locus. Poor metabolizers are homo-
zygous for an autosomal recessive allele and usually have ratios
greater than 20 (ref. 125). Oxidation polymorphism has been iden-
tified in the metabolism of the lipophilic beta-blockers metopro-
lol (refs. 122-124, 127, 128), bufuralol (refs. 129-132), timolol
(ref. 133) and bopindolol (ref. 134). For propranolol only the 4-
hydroxylation metabolic pathway is genetically controlled (refs.
135, 136). There was no relationship between the ratio of debriso-
quine to 4-hydroxydebrisoquine and the pharmacokinetics or phar-
macodynamics of atenolol (ref. 133).

In poor metabolizers the plasma levels and the accompanying
pharmacodynamic effects of a beta-blocker, in whose metabolism
genetic polymorphism has been identified, may be higher than those
of extensive metabolizers (refs. 121, 137). The poor metabolizers
constitute a single subgroup with respect to a propensity to deve-
lop exaggerated responses, untoward drug effects or therapeutic
failure when the oxidized metabolites are the major active pro-
ducts (ref. 138).

In the following section some data illustrating the polymor-
phic oxidative metabolism of metoprolol, bufuralol and timolol
will be reviewed.

The plasma level of metoprolol after a single oral dose was
higher and its elimination half-life was longer in poor metabo-
lizers than extensive metabolizers (refs. 122, 139, 140). A close

relationship has been found between the oral clearance of metoprolol and the clearance of debrisoquine (ref. 122) or sparteine (refs. 141, 142)(the oxidation of debrisoquine and sparteine seems to be under common genetic control). It was suggested that the same cytochrome P-450 isozyme catalyses the metabolism of metoprolol and sparteine (ref. 142). In a large population the frequency distribution of plasma metoprolol concentrations (the metoprolol concentration in plasma 3 h after an oral dose of metoprolol) and of the ratio of metoprolol to α-hydroxymetoprolol in urine was bimodal and correlated with the debrisoquine to 4-hydroxydebrisoquine ratio (refs. 124, 139, 143). It has been shown that the polymorphism in metoprolol oxidation is caused by the inherited absence or functional deficiency of a particular cytochrome P-450 isozyme (refs. 144, 145). The isozyme has substrate stereoselectivity, which means that it preferentially metabolizes a particular enantiomer of metoprolol (ref. 127). Quinidine inhibits metoprolol metabolism, mimicking the poor-metabolizer phenotype. No effect of quinine was observed in poor metabolizers. In extensive metabolizers quinine produced a three-fold increase in total and (−)-metoprolol concentration (ref. 146).

The metabolism of bufuralol to the carbinol (1 -hydroxybufuralol) is under the same genetic control as the 4-hydroxylation of debrisoquine (refs. 129-132, 147). In extensive metabolizers the systemic availability of bufuralol was 43%. Poor metabolizers were characterized by a considerable increase in systemic availability due to a corresponding decrease in hepatic first-pass metabolism. It was concluded that the target of the genetic variation is hepatic handling of the drug (ref. 140). In poor metabolizers (8% of the sample) the plasma bufuralol concentrations were very high and the metabolite concentrations were low; the carbinol concentrations in poor metabolizers were far lower than in extensive metabolizers (ref. 132). In another study, 3 h after administration of racemic bufuralol the plasma (−)/(+)- isomer ratio for unchanged bufuralol was 1.84 in extensive metabolizers, indicating preferential clearance of the (+)-isomer. Poor metabolizers were characterized by impaired 1 - and 4-hydroxylation, with almost total abolition of the stereoselectivity of these reactions (ref. 148).

For timolol a correlation between the plasma AUC and the ratio of debrisoquine to 4-hydroxydebrisoquine was found and the mean of the AUC values for timolol was higher in the poor metabolizers than in the extensive metabolizers (ref. 133). In hyper-

tensive patients poor metabolizers had longer elimination half-
lives of timolol than extensive metabolizers (ref. 149).

The elimination of bopindolol was slightly but significantly
prolonged in poor metabolizers (ref. 148).

It may be concluded from these and other data not cited here
that the genetic status is a major source of inter-individual
variation in the plasma concentration of drugs that undergo oxida-
tive metabolism (ref. 132).

2.9 STEREOSELECTIVE DISPOSITION OF BETA-BLOCKERS IN MAN

In the molecule of all clinically used beta-blockers a centre
of asymmetry is present, related to the presence of four different
groups attached to one carbon atom. This results in the occurrence
of two stereoisomers, enantiomers characterized by their mirror
image relationship. Most of the beta-blockers are administered as
racemic mixtures of the d- and l-isomers. Stereoselectivity in
drug action implies that in a mixture of isomers only one is the-
rapeutically active (ref. 150). Exceptionally, a clinically used
beta-blocker preparation contains only the therapeutically active
isomer. The stereospecific or stereoselective interactions between
the chiral macromolecules of the body and the stereoisomers of the
drug result in stereoselective pharmacokinetics and pharmacodyna-
mics. The study of stereoselective pharmacokinetics of beta-blo-
ckers ran parallel with the development of stereoselective methods
of analysis (Chapter 7). Some of the most important data on the
stereoselective pharmacokinetics of beta-blockers in man can be
summarized as follows.

After administration to man of most beta-blockers in a race-
mic mixture, the concentration of the l-isomer is higher than that
of the d-isomer. This has been found for propranolol (refs. 151-
157), alprenolol (ref. 158), metoprolol (ref. 127, 158), bufuralol
(ref. 159), and moprolol (ref. 160). For pindolol the maximum
concentration of d- and l-pindolol were identical in each sub-
ject; however, the plasma concentrations of d- and l-pindolol ten-
ded to diverge at later times, with concentrations of l-pindolol
being slightly higher than those of d-pindolol (ref. 161). In the
disposition of either acebutolol or its metabolite diacetolol in
man, no important stereoselectivity could be observed. This may be
due to the fact that acebutolol is metabolized by hydrolysis and
N-acetylation, whereas the other beta-blockers are primarily meta-
bolized by oxidation (ref. 162).

The differences between the plasma concentrations of the l- and d-isomers may be of interest as the beta-adrenergic blocking action is clearly stereoselective, with the l-isomer being pharmacologically active. However, owing to the large therapeutic index of beta blockers the clinical significance of these differences is questionable.

In another study (ref. 69), in order to detect stereoselective pharmacokinetics of the racemates of three beta-blockers, plasma concentrations were monitored by HPLC and by means of a subtype-selective receptor assay, using a $beta_1$-adrenoceptor preparation from the rat salivary gland. A plot of log (concentration detected by HPLC) vs. log (concentration detected by receptor assay) was constructed. In the case of stereoselective bioavailability and/or clearance, one would expect a deviation from the line of identity in favour of the physico-chemical detection method if the active isomer (eutomer) shows a lower bioavailability and/or higher clearance than the inactive isomer (distomer). Otherwise, the deviation would be in favour of the receptor assay. For all the beta-blockers investigated, propranolol, atenolol and bisoprolol, no significant stereoselective elimination characteristic could be detected.

The different dispositions of the l- and d-isomer may have various causes.

First, stereoselective plasma binding should be mentioned, possibly leading to different kinetic behaviours of the two enantiomers. Plasma binding of the propranolol enantiomers differed, the unbound fraction of (-)-propranolol being smaller than that of (+)-propranolol. The (-)/(+)-propranolol ratio for the unbound fraction, a measure of stereoselectivity, was 0.86 ± 0.02. The stereoselectivity was greater at higher total binding. The binding to α_1-acid glycoprotein was stereoselective for (-)-propranolol with a (-)/(+)-propranolol ratio for the unbound fraction of 0.79 ± 0.01, whereas (+)-propranolol was bound to a greater extent to human serum albumin. The distribution volume and extravascular distribution volume should be smaller for (-)-propranolol than for (+)-propranolol (ref. 163), as actually found in a study on human volunteers (ref. 156).

Second, stereoselective clearance came into play in four subjects continuously treated with oral propranolol in daily doses of 160, 240 and 320 mg; the intrinsic clearance of (S)(-)-propranolol was always lower than that of the (R)(+)-enantiomer and the termi-

nal elimination half-lives of (S)(-)-propranolol and its glucuro-
nide conjugate were longer than those for the (R)(+)-enantiomer.
The formation of glucuronide conjugates of propranolol was a satu-
rable process and the V_{max}/K_m ratio was greater for the (S)(-)-
propranolol than for the (R)(+)-enantiomer (ref. 154). After an
intravenous dose of racemic propranolol the systemic clearance and
the apparent distribution volume were greater for (+)- than (-)-
propranolol (refs. 156, 157). The higher systemic clearance of
(+)-propranolol suggested stereoselective hepatic elimination
(ref. 156). The oral bioavailability in man was 45% greater for
(-)- than (+)-propranolol. Stereoselectivity in bioavailability
implies stereoselectivity in intrinsic hepatic drug-metabolizing
enzyme activity (ref. 157). No difference was found in the rates
of absorption of the two isomers of propranolol (ref. 151). Ste-
reoselectivity in renal elimination was reported after administra-
tion of a racemic mixture of pindolol to human volunteers. The
renal clearance of l-pindolol was greater than that of d-pindolol.
Simultaneously, stereoselectivity in pindolol elimination by non-
renal routes was suggested (ref. 161).

Stereoselectivity in the metabolism of beta-blockers control-
led by genetic polymorphism has been outlined in Section 2.8.

2.10 INTER- AND INTRA-SUBJECT VARIABILITY OF THE PHARMACOKINETICS OF BETA-BOCKERS

Information on the variability of parameters describing the
pharmacokinetics of a drug is of primary importance for the clini-
cian who establishes and carries out the patient's individual
therapeutic regimen.

The analytical chemist confronted with the determination of
beta-blockers in biological material, mainly serum or plasma,
should also have an adequate knowledge concerning the range of
concentrations which may be expected in the sample being analysed
(see Section 2.3) and concerning the variability of these levels
within an individual (intra-subject variability) and between indi-
viduals. It does not seem necessary to stress that the inter-
subject variability is one of the factors determining the variabi-
lity of the derived pharmacokinetic parameters, characteristic for
a population sample. The purpose of this section is to assemble
some data concerning the variability of the concentrations of beta
blockers in plasma or serum. It should be emphasized that some
published data, which will be reviewed, are semiquantitative and

as a whole very heterogeneous with respect to the statistical methods used and the matter treated. The author's intention was not to make some kind of inter-drug comparison, which can be done only under strictly defined experimental conditions, but rather to provide fundamental information about the problem and sources of data for a more detailed study.

2.10.1 Practolol
Between 2 and 7 hours after oral administration of the drug the blood levels varied little more than two-fold either within or between subjects (ref. 49).

2.10.2 Nadolol
Inter-individual variations in plasma nadolol levels were in accordance with those of other beta-blockers that are devoid of a first-pass effect (ref. 80).

2.10.3 Atenolol
A 2- to 5-fold variation in plasma concentration between individual subjects at 24 h in the dose range studied was reported (ref. 43). Correspondingly, in another study a 4-fold variation in blood levels between individuals receiving atenolol orally was found (refs. 164, 165).

2.10.4 Sotalol
The plasma levels of sotalol measured 2 hours after oral administration of 25 – 800 mg never showed more than a 6-fold variation between different subjects (ref. 47).

2.10.5 Metipranolol
Less than a 3-fold difference in the AUCs and less than a 2-fold difference in the half-lives for metipranolol in human volunteers were reported (ref. 74).

2.10.6 Metoprolol
Large inter-individual differences in the bioavailability of metoprolol were observed in both young and elderly groups. The variation of the peak concentration was 13- and 4-fold and that of the AUC was 43- and 10-fold in the young and elderly, respectively (ref. 166). The inter- and intra-subject variabilities for metoprolol were higher than those for oxprenolol and acebutolol

(ref. 167).

2.10.7 Esmolol

Variations with a coefficient of variation of 46.4% for $t_{1/2\ beta}$, 31.3% for clearance and 75.1% for V_c were reported. The peak plasma concentration of esmolol at the steady state had a coefficient of variation of 27.1%. It was stressed, however, that even a 300% range of the elimination $t_{1/2}$ with an extremely short-acting drug such as esmolol would not have a major clinical impact. The need for dosage regimens based on effect and individual needs was emphasized (ref. 168).

2.10.8 Tolamol

Between subjects there was a 4-fold variation in maximum tolamol concentration and an 8-fold variation in the AUCs after the same oral dose (ref. 46).

2.10.9 Pindolol

A low variability of pindolol plasma concentrations was reported; the variation in steady-state concentrations was small in each individual and also between individuals (ref. 76). Peak plasma levels of pindolol 80 min after oral administration showed up to 4-fold variations after identical doses (ref. 169).

2.10.10 Oxprenolol

The intra-subject variability was considered to be low owing to the observed repeatability of the plasma curve when oxprenolol was given to healthy subjects under controlled conditions (ref. 170). It follows from another study that oxprenolol concentrations are less subject to variability than those of metoprolol and propranolol (ref. 167). On the other hand, the variability in the plasma concentration of oxprenolol between patients on oral treatment was reported to be large at all dose levels and with both normal and sustained-release formulations. It was so wide as to negate any attempt to derive a prescription based on the time and magnitude of the last oral dose. Thus any general prescription would have wide confidence limits (ref. 73).

2.10.11 Acebutolol

The blood clearance of acebutolol had a coefficient of variation of 14% for the group studied (ref. 83).

44

2.10.12 Alprenolol

The steady-state plasma concentration of alprenolol in pa-
tients treated for a prolonged period varied 25-fold between pa-
tients who received identical doses (ref. 171).

2.10.13 Propranolol

The views about the variability of propranolol pharmacokine-
tics in man differ considerably. At one extreme wide inter- and
intra-individual variations in serum propranolol levels have been
reported (ref. 172). In line with this, significant inter-subject
variations in the peak plasma concentration and in the AUC for
propranolol were reported. For the AUC of propranolol, values of
781 and 3148 for the inter-dose and inter-subject unbiased varian-
ce, respectively, were found. For comparison the corresponding
data for befunolol were 49 and 148.9 (ref. 41). It follows from
another report that at each of three propranolol doses, 40, 80 and
320 mg daily, approximately a 20-fold range in the steady-state
plasma propranolol concentrations was observed (ref. 24).

On the other hand, there are data showing less variation in
propranolol pharmacokinetics. At the steady state after 40-320
mg/day, in the C_{ss} of propranolol in plasma there was a 1.8- to
2.6-fold difference between four subjects (ref. 92). Chidsey (ref.
38) found only a 4-fold difference between four subjects. In a
further report, the variability of plasma propranolol concentra-
tion found could be considered small. It varied within subjects
from 27 to 36% and between subjects from 19 to 28% at the various
sampling times. Pharmacodynamic effects showed a similar reprodu-
cibility (ref. 173). The last study (ref. 97) to be mentioned was
made during chronic propranolol therapy. Propranolol doses ranged
from 40 to 960 mg/day (every 6 h). The between-patient variation
in plasma was much smaller than had previously been reported, in
spite of the fact that the patient population studied was hetero-
geneous and that numerous other drugs were concomitantly used with
propranolol. A maximum 3-fold variation in peak plasma concen-
trations at the steady state was observed at the 40-mg dose level,
which decreased linearly with dose to only a 1.3-fold variation at
doses exceeding 600 mg/day. The 3-fold between-patient variation
at low doses is believed to be associated with a high presystemic
elimination at these doses, a process that may become partially
saturated at higher doses, leading to a dose-dependent decrease in

the between-patient variability.

2.11   PHARMACOKINETICS OF BETA-BLOCKERS IN PREGNANCY

Pregnancy is accompanied by changes in the neural and hormonal regulation of the function of many organs. It may be expected that changes in the glomerular filtration rate will be reflected in the elimination of non-metabolized, hydrophilic beta-blockers, and changes in drug metabolism in the elimination of the more lipophilic, extensively metabolized beta-blockers.

The following reports were available. The pharmacokinetics of atenolol in pregnant women did not differ from that of the non-pregnant women. The average maternal blood concentration was 0.26 μg/ml and that of the cord blood 0.22 μg/ml (refs. 174, 175). Plasma levels of labetalol in hypertensive pregnant women were usually lower than those reported for the non-pregnant state at a comparable dose (ref. 176). In another study, the clearance of labetalol in pregnancy remained unchanged (ref. 177). The pharmacokinetics of propranolol, propranolol glucuronide, 4-hydroxypropranolol and 4-hydroxypropranololglucuronide were not significantly altered by pregnancy. However, the kinetics of naphthoxylactic acid appeared to be altered. The formation of naphthoxylactic acid by N-dealkylation of propranolol and further oxidation appears to be competitively inhibited by unidentified substances, perhaps endogenous steroids (ref. 59). For metoprolol an increased hepatic clearance was reported, which led to lower plasma levels of metoprolol during pregnancy compared with the post-delivery period (ref. 178).

2.12   PLACENTAL TRANSFER OF BETA-BLOCKERS

Both the pharmacokinetic and pharmacodynamic aspects of the problem of placental transfer of beta-adrenoceptor blocking agents has been reviewed recently by Boutroy (ref. 20). From the data presented it follows that foetal plasma concentrations (F) are often close to those of the mother (M), the ratio F/M being between 0.7 and 1.0, except for oxprenolol. After birth the newborn eliminates the drug, but generally more slowly than does an older child or an adult, as shown in the case of metoprolol (ref. 179). In women treated with this drug for hypertension in pregnancy, peak concentrations of metoprolol and α-hydroxymetoprolol were reached 60 - 120 min after dosing in maternal plasma, whereas in the amniotic fluid their levels continued to increase up to the

end of the study and after $4 - 5$ h they were substantially
higher than in the plasma. No measurable concentrations of meto-
prolol were found in two of the newborns 2 h after delivery. In
the remaining four neonates the 2-h post-delivery concentration
exceeded the corresponding cord plasma level. In two of these
newborns the metabolite levels continued to increase for 5 h and
in one the highest blood concentrations of the metabolite was
found 20 h after birth. Lindeberg's study presented a hypothesis
regarding the dynamics of drug and metabolite exchanges between
mother, foetus and amniotic fluid and provided an explanation of
the temporary increase in the neonatal plasma levels of the drug
immediately after birth.

2.13 EXCRETION OF BETA-BLOCKERS INTO HUMAN BREAST MILK

Beta-blockers have been increasingly administered to pregnant
and lactating women for the treatment of hypertension. Depending
on their physico-chemical properties, beta-blockers are excreted
in breast milk and the nursed infant may be exposed to their
effects. The passage of a drug into the milk depends on its lipid
solubility, its degree of ionization and its protein binding (ref.
180). The clinically used beta-blockers are bases and their pK_a
values lie within a relatively narrow range of 9.2-9.75 (see Table
3.1). On the other hand, the lipid solubilities and the protein-
binding characteristics of the beta-adrenoceptor blocking drugs
vary considerably. The pH of breast milk was reported to range
from 6.35 to 7.65, with an average of 7.08 (ref. 181). According
to the pH partition principle, for basic drugs a milk/plasma con-
centration ratio >1 could be expected. However, the following sur-
vey of published data shows ratios varying within a broad range,
evidently due to a greater complexity of relevant factors.

2.13.1 Metoprolol

The drug concentration in breast milk was higher than that in
serum or plasma and the resulting mean AUC values were about three
times greater in milk than in serum or plasma (refs. 182-184). The
plasma concentrations of metoprolol in infants were negligible or
below the limit of detection. Exposure of the infant to metoprolol
can be minimized if nursing is not undertaken earlier than 3-4 h
after dose intake (ref. 183).

2.13.2 Atenolol

The ratio between the area under the milk concentration vs. time curve and the area under the plasma concentration vs. time curve in the mother varied between 1.1 and 3.1 (ref. 183) or 1.5 and 6.8 (milk/serum)(ref. 182). In other studies a mean value of 1.69 for the ratio breast milk/plasma was found (ref. 185) or 2.9 4 h after atenolol intake (ref. 186). The high values of the ratios found may be rationalized by the high pK_a value of atenolol (9.5) causing accumulation of atenolol in breast milk as a result of "ion trapping" (ref. 186). In contrast to the above data, a mean milk/blood value of 1:1.3 was reported by Thorley (ref. 175).

It was thus concluded that maternal ingestion of atenolol in conventional doses poses no threat to the breast-fed infant (refs. 185, 186).

2.13.3 Oxprenolol

The concentration of oxprenolol in breast milk varies from 15% to 45% of those in plasma, with a mean value of about 30% (ref. 29). In another study the mean milk to plasma ratio was 0.29 ± 0.14 (ref. 187). The amount of oxprenolol ingested by the infant via breast milk would be clinically unimportant (ref. 29).

2.13.4 Timolol

The mean milk to plasma ratio of timolol was 0.8 ± 0.21 (ref. 187).

2.13.5 Mepindolol

The average milk/plasma drug concentration ratio was found to be 0.38. Plasma levels in the newborns were below the detection limit of 1 ng/ml, except for one baby in whom 2 and 5 ng/ml were found (ref. 188).

2.13.6 Propranolol

A dose-dependent entry of propranolol into milk, with a milk-to-plasma ratio of roughly 1, was reported (ref. 189). In other studies a ratio of 0.63 - 0.35 (ref. 191) or 0.36 (ref. 190) was found.

2.14 EFFECT OF FOOD ON THE PLASMA LEVEL OF BETA-BLOCKERS

To treat this problem one has to keep in mind the physico--chemical differences between the various beta-blockers and the various stages of the whole process involved between the dis-

appearance of the drug from the lumen of the gastrointestinal tract and its appearance in the systemic circulation. It can be stated that the lipophilic beta-blockers are rapidly and completely absorbed from the gastrointestinal tract, but are exposed to first-pass metabolism of varying extent. On the other hand, incomplete absorption from the gastrointestinal tract, no first-pass elimination and little or no metabolic elimination are characteristic of the hydrophilic beta-blockers (ref. 11). Food interferes with drug availability in a distinct manner at various stages of the process. Different responses may therefore be expected after oral and intravenous administration. Thus, food increases the plasma concentrations of orally administered propranolol and metoprolol (refs. 192-195) and the bioavailability of propranolol (refs. 196, 197) and labetalol (refs. 198, 199). When the diet was altered from high carbohydrate/low protein to low carbohydrate/high protein, the oral clearance of propranolol increased by $74\pm20\%$ with no change in the plasma half-life or plasma binding (ref. 200). On the other hand, during a steady-state infusion of propranolol there was a rapid decrease (mean 35%) in the blood propranolol concentration within 5 min of feeding and the levels were reduced for 30 min (ref. 201). In accord with this, food intake increased the systemic clearance (after intravenous administration) of propranolol by 38% with no change in $t_{1/2}$ or the apparent distribution volume (ref. 197).

The increased bioavailability of lipid-soluble beta-blockers after administration of food can be explained on the basis of an increased liver blood flow and the fact that hepatic extraction of drugs is to a great extent governed by diffusion of the drug from blood to tissue overcoming a diffusion barrier. A diffusion process would be expected to become rate-limiting in the extraction of a drug, especially when the blood flow-rates are very high, as in the liver (ref. 196). A very short duration of the food-induced hyperaemia response may also be the reason why food has no effect on the oral plasma concentrations of propranolol when the drug is given in a slow-release formulation (ref. 202).

Food had little or no effect on the kinetics of several beta-blockers: oxprenolol (ref. 203), bisoprol (ref. 204), bevantolol (ref. 81), acebutolol (ref. 205), timolol (ref. 206) and pindolol (ref. 207). In another study on pindolol, food appeared to increase the speed of its absorption, leading to a slightly earlier and higher maximum plasma concentration, but this observation

appears to be of no clinical relevance (ref. 208).

The interference of food with beta-blockers may take place before or directly in the absorption process. The disappearance rate of oxprenolol from the jejunum was unaffected by endogenous secretions, but the intestinal absorption rate was markedly increased in the presence of nutrients (ref. 209), and similarly the absorption rate of metoprolol perfused in the jejunum in a saline solution appeared to be lower than that observed after gastric administration of the drug incorporated in a meal (ref. 210).

Finally, large volumes of fluid delayed but did not affect the extent of hydrophilic sotalol absorption. Food, especially milk, decreased the bioavailability of the drug and an interaction with calcium seems to be the major reason for the reduced absorption (ref. 211). Food intake shortened the time to reach the peak concentration of another hydrophilic beta-blocker, atenolol, but caused a reduction in AUC values due to impairment of absorption (ref. 193).

2.15 PHARMACOKINETICS OF BETA-BLOCKERS IN ELDERLY SUBJECTS

Ageing may be accompanied by changes in the function of those organs which are involved in the elimination of beta-blockers from the organism. The greater occurrence of cardiovascular diseases at advanced compared with young age groups results in an enhanced consumption of beta-blockers in the elderly. Consequently, the pharmacokinetics of beta-adrenoceptor blocking drugs in the aged has attracted much attention.

Negligible effects of age on the pharmacokinetics of metoprolol were reported in several studies (refs. 166, 212-215). They can be specified as a lower availability of metoprolol in the elderly compared with the control group of young volunteers (refs. 214, 215) and as a significantly longer time to peak concentration in the elderly (ref. 166).

The plasma propranolol level in a group of elderly subjects was substantially higher than the corresponding level in a group of young subjects (refs. 216-219). On the other hand, in other studies concerned with a similar topic no change in the pharmacokinetics of propranolol with ageing was observed (refs. 220, 221).

The reduced glomerular filtration rate in aged people may result in a decreased elimination rate of beta-blockers excreted in unmetabolized form via the kidney. Thus, the terminal half-life

of sotalol was prolonged and its renal clearance reduced in elder-
ly compared with young individuals (ref. 222).

There was no uniform effect of age on the kinetic parameters
of atenolol. C_{max} and t_{max} remained in the normal range. The mean
AUC increased with age, but there were also normal and even lower
values in elderly patients. The elimination half-life remained
unchanged with age (ref. 223).

The decreased glomerular filtration rate may be the operating
mechanism of the reduced elimination of some metabolites having a
more polar character than the parent drug. Thus, in the elderly,
the mean plasma concentration of α-hydroxymetoprolol was about
twice that of the parent drug, whereas the opposite was true of
the control group (ref. 215). Similar results were obtained in
another study investigating the effect of age on the kinetics of
metoprolol and its metabolites (ref. 214).

Higher blood levels of acebutolol in elderly compared with
young people were reported (refs. 224, 225). A reduced first-pass
effect in aged individuals was suggested as the underlying mecha-
nism (ref. 225).

For diacetolol, a metabolite of acebutolol, the terminal
half-lives in the elderly were increased (refs. 224, 225). The
half-life of diacetolol is correlated with serum creatinine and
the decreased renal clearance in older patients was considered to
be the most plausible explanation (ref. 225).

For celiprolol no excessive accumulation was reported in the
elderly population with normal renal and hepatic function (ref.
88).

2.16 EFFECT OF DISEASE ON THE CLINICAL PHARMACOKINETICS OF BETA-
BLOCKERS

The pharmacokinetic behaviour of a drug is given by the sum
of several partial processes: absorption of the drug, its distri-
bution in the body, its biotransformation and excretion in unchan-
ged or metabolized form. It is obvious that a certain pharmaco-
kinetic pattern will depend, among other factors, on normal fun-
ctioning of many organs: the gastrointestinal tract for absorption
of orally administered drugs, the heart for transport of drugs by
circulating blood, constituents of blood and of peripheral tissues
for distribution processes, the liver and other organs with meta-
bolic activity for biotransformation of drugs and the kidney,
liver and gut for their excretion.

2.16.1 <u>Interference with renal elimination</u>

Drugs are eliminated from the organism predominantly by metabolism and, if not metabolized, by excretion in unchanged form. Excretion of unmetabolized drugs may occur by various routes, but urinary excretion is the most common. Drug metabolites are also frequently excreted by this route. When renal function is impaired, an accumulation in the body of drugs eliminated by the kidney can be expected or of drug metabolites if renal excretion is involved in their elimination. Many data show that the excretion rate of beta-blockers that do not undergo metabolism in the body can be correlated with some quantitative index of renal function. Glomerular filtration rate is the most commonly employed index of kidney function with respect to excretion of drugs. It can be estimated by measurement of creatinine, inulin or iothalamate clearance (for details see Brater (ref. 226)).

The elimination rate of unmetabolized or slightly metabolized beta-blockers correlates well with the glomerular filtration rate. This holds both for population samples with normal kidney functioning and for patients with impaired renal function, and was documented under the following references: atenolol (refs. 43-45, 227-229); carteolol (refs. 230, 231); practolol (refs. 232, 233); sotalol (refs. 222, 234, 235); nadolol (ref. 236). For some drugs of this group of beta-blockers renal clearances greater than the glomerular filtration rate were reported, suggesting some tubular secretion (practolol (ref. 232, 233), carteolol (ref. 230)).

The other extreme represents drugs eliminated wholly or predominantly by metabolism. It can be expected that their total body clearance will be independent of kidney function. This may be documented by the following reports: timolol (refs. 237, 238), penbutolol (ref. 112), mepindolol (ref. 239), metoprolol (ref. 240), metipranolol (ref. 241) and propranolol (ref. 242).

Many beta-blockers are eliminated by both renal excretion and metabolism. These two distinct mechanisms combine in various proportions depending on the chemical structure of the drug molecule and the extent of its biotransformation. It may be expected that the total body clearance of this group of drugs reflects both hepatic and renal impairment. Pindolol may serve as an example. Compared with patients with normal renal function, patients with chronic renal failure exhibited /1/ unchanged transfer rate constants (between compartments of the two-compartment model) and

distribution volumes and /2/ decreased total body clearance with
decreased renal clearance and unchanged non-renal clearance (refs.
243-245). Similarly to unmetabolized beta-blockers, the renal
clearance of this group of drugs was found to correlate with crea-
tinine clearance (for pindolol (refs. 243, 244, 246) and acebuto-
lol (ref. 247)). Even for propranolol, which is excreted in the
urine in unchanged form in less than 1% of the dose on average, a
correlation between renal clearance of free propranolol and crea-
tinine clearance was obtained (ref. 248).

Not only are changes in elimination associated with the mal-
functioning kidney, but also alterations in the kinetics of the
absorption process have been reported for some beta-blockers to
accompany renal impairment. Thus patients with chronic renal
failure exhibited a decreased fraction of the dose of pindolol
effectively absorbed with a concomitantly increased initial rate
of absorption which was inversely correlated with the creatinine
clearance (refs. 243-245, 249). For propranolol the maximal con-
centration was higher and it developed earlier in patients with
renal impairment than in controls and simultaneously a smaller
first-pass effect was observed (ref. 250). However, a delay of
peak propranolol plasma levels was also reported in patients with
impaired renal function (ref. 251).

Metabolites formed as a result of the drug biotransformation
process are normally more polar than the parent drug which favours
their renal excretion. Therefore, renal impairment may be reflec-
ted in the rate of excretion of metabolites, while elimination of
the parent drug remains at a normal level. Thus the kinetics of
unchanged penbutolol were not altered by renal impairment, whereas
the elimination rate of penbutolol glucuronide was related to the
glomerular filtration rate (ref. 112). Similarly, the plasma level
of acebutolol was not influenced by changes in renal function
whereas that of the metabolite N-acetylacebutolol was considerably
increased in preuraemic patients (refs. 247, 252). The urinary
excretion of propranolol metabolites was also directly related to
renal function (refs. 251, 253). The α-hydroxymetoprolol metabo-
lite of metoprolol may accumulate in renal disease (ref. 240).

2.16.2 Interference with hepatic elimination

Under certain circumstances, the liver may be the site of a
broad spectrum of pathological processes. These may alter one or
more of the innumerable liver functions. The routes which lead to

changes in the pharmacokinetics of drugs as a result of a mal-
functioning liver are reduction in blood flow, extra- or intrahe-
patic shunting of blood, hepatocyte dysfunction, quantitative and
qualitative changes in serum proteins and changes in bile flow
(ref. 254). This heterogeneity of the pathophysiological mecha-
nisms leads to a great variability of changes in drug kinetics as
a result of a diseased liver. Whereas in many instances renal
elimination of drugs correlates with the glomerular filtration
rate, for hepatic drug elimination a single correlating factor of
liver function will not presumably be established.

For beta-blockers which are extensively excreted in unchanged
form by the kidney, little effect of liver disease on their phar-
macokinetics can be expected. Thus the pharmacokinetic parameters
for atenolol (refs. 255-257) and sotalol (ref. 256) did not differ
between patients with chronic liver disease and healthy volun-
teers. On the other hand, a tendency for an increased distribution
volume of atenolol could be observed in subjects with liver
disease compared with normal volunteers (ref. 255). Similarly, in
patients with cirrhosis no differences in the pharmacokinetics of
acebutolol or of its metabolite diacetolol were found in compari-
son with healthy volunteers (ref. 258). Metipranolol has no hepa-
tic first-pass effect and its total clearance remains largely
uninfluenced even in severe liver damage (ref. 259). The liver
plays only a minor role, if any, in the elimination of esmolol
(ref. 260). No significant differences in the main pharmacokinetic
parameters were detected between control subjects and those with
hepatic disease for either esmolol or its acid metabolite (ref.
261).

For beta-blockers eliminated from the body largely by metabo-
lism, altered kinetics in patients with liver diseases were repor-
ted. For betaxolol a reduced intrinsic clearance led to an
increase in the elimination half-life (ref. 262). In patients with
liver cirrhosis the elimination half-life of bisoprolol increased
to 13.5 h (normally 10 h), and the total body clearance decreased
to 10.8 l/h (normally 14.2 l/h) (ref. 263). Patients suffering
from cirrhosis showed a correlation between total body clearance
of antipyrine and clearance of pindolol (ref. 264). For metoprolol
the mean total concentrations were increased in cirrhosis and the
mean fraction of the drug available was 84% in patients and 50% in
the control group (refs. 240, 265). Similarly, propranolol first-
pass elimination and systemic clearance decreased in cirrhotic

patients (refs. 257, 266, 267) and the free fraction of proprano-
lol increased (ref. 266). An altered disposition of (+)-proprano-
lol in cirrhotic patients with serum albumin concentrations of
less than 30 g/l was reported (ref. 268). The mean elimination
half-life in these patients was almost eight times that in the
control group, together with a 2-fold increase in the distribution
volume and a 4-fold decrease in systemic clearance. In a more
recent study (ref. 267), in patients with cirrhosis (serum albumin
<30 g/l) propranolol remained detectable in the plasma 24 h after
a single 20 mg dose and high steady-state concentrations (mean
266.5 ng/ml) were observed during regular dosing.

2.16.3 Thyroid disease

Beta-blockers are frequently used in the management of thy-
roid dysfunction. Hyper- or hypothyroidism is associated with fun-
ctional changes of the cardiovascular, hepatic and renal system
which may alter the disposition of drugs. This topic was reviewed
in detail by Feely (ref. 269) and here only some facts essential
to the analytical chemist working in this field will be mentioned.

The clearance of the hydrophilic renally excreted beta blo-
ckers is unaltered in hyperthyroidism. This has been demonstrated
for sotalol (ref. 270), nadolol (ref. 271) and atenolol (ref.
272).

For propranolol, trough and peak serum levels were lower when
the patients were in a thyrotoxic state than when they were in a
euthyroid state (ref. 271). Correspondingly, in another study thy-
rotoxicosis was associated with doubling of both oral and systemic
clearances of unbound propranolol, which resulted in an approxima-
tely 50% reduction in blood concentrations after oral doses (ref.
273). Similarly to propranolol, the clearance of metoprolol was
increased in hyperthyroidism (ref. 272). These changes were attri-
butable to increases in hepatic blood flow and drug-metabolizing
activity of the liver (ref. 269). In another study (ref. 274), on
the other hand, no substantial difference in propranolol elimina-
tion from the plasma of hyperthyroid and euthyroid patients or
healthy subjects was found.

2.16.4 Miscellaneous diseases

Of other diseases which may interfere with the disposition of
beta-blockers diseases and other pathophysiological states accom-
panied by alterations in the constituents of plasma proteins

should be mentioned.

In patients with ischaemic heart disease a higher plasma ox-
prenolol concentration/time profile was observed than in volun-
teers (ref. 73). The total body clearance of propranolol was in-
versely related to the basal diastolic arterial pressure in hyper-
tensive patients (ref. 275). For pindolol kinetics no effect of
hypertension was reported (ref. 207).

α_1 -Acid glycoprotein (AGP), the principal drug binding protein
in orosomucoid, is an important determinant in the binding of a
variety of basic drugs (refs. 276, 277). A number of pathophysio-
logical states are accompanied by an increase in AGP in plasma:
rheumatoid arthritis, Crohn's disease, myocardial infarction,
burns, trauma, cancer, renal transplantation, postoperative
states, ulcerative colitis. Some beta-blockers have been shown to
bind mainly to the AGP in serum: alprenolol (ref. 278), proprano-
lol (refs. 279-283). In the binding of propranolol the free frac-
tion was shown to correlate negatively with the AGP concentration
(ref. 284). This is important clinically as the beta-adrenoceptor
blocking effects of propranolol correlate well with free (unbound)
rather than total plasma concentration (ref. 285). The following
data should illustrate some clinically relevant situations. Signi-
ficantly higher AGP concentrations and a reduced unbound propra-
nolol fraction were observed in the elderly with acute disease
compared with the elderly controls (ref. 286). The rise in AGP
following myocardial infarction was associated with an increase in
plasma propranolol binding (ref. 13). Elevated blood levels of
oxprenolol were seen in volunteers who developed mild upper respi-
ratory tract infection or in patients with rheumatoid arthritis
compared with normal volunteers (ref. 170). The percentage of free
propranolol in plasma was decreased in patients with Crohn's
disease, inflammatory arthritis and chronic renal failure with
superimposed inflammatory disease compared with healthy controls
(ref. 284). In cancer patients the AGP concentrations were twice
those in controls and propranolol binding correlated strongly with
AGP concentration (ref. 287).

These findings emphasize that free drug concentrations should
be considered in the design of dosage regimens for AGP-bound beta-
-blockers in pathophysiological states accompanied by alterations
in the concentration of the "acute-phase plasma proteins" (ref.
288).

The kinetics of metoprolol were unaltered in inflammatory di-

sease (refs. 289, 290), whereas for atenolol reduced absorption in the gastrointestinal tract and enhanced elimination from plasma were observed during inflammatory disease (ref. 289).

In this context it should be noted that impaired liver and kidney function may also lead to serious alterations in the concentration of plasma binding proteins. The changes in the kinetics of drugs, including beta-blockers, in such states have already been thoroughly reviewed (refs. 276, 291).

Alterations in various physiological functions and in the proportion of various constituents of the body are characteristic of obese subjects. Modified dispositions of beta-blockers in obese individuals can be expected. In one study the distribution volume and half-life of propranolol were greater in obese volunteers than in normal volunteers (ref. 292), whereas in a more recently published study (ref. 293) obese subjects showed an increase in propranolol AUC and a decrease in distribution volume in the steady-state and a decrease in total clearance. Altered hepatic function and tissue blood flow were proposed as an explanation for these changes.

In undernourished subjects with or without infection the level of AGP was found to be elevated compared with controls. In the same subjects the percentage of free drug was diminished (ref. 294).

2.17 INTERACTION OF DRUGS WITH THE PHARMACOKINETICS OF BETA-BLOCKERS

Similarly to the interaction of food with the kinetics of beta-blockers, the effects resulting from the drug interactions will largely be dependent on the distribution and elimination profile of the beta-blocker in question.

Beta-blockers are often used concurrently with drugs acting on the cardiovascular system. No pharmacokinetic interaction between nifedipine and metoprolol or atenolol was found (refs. 295, 296). Hydralazine affected only the kinetics of beta-blockers with a substantial first-pass loss. Thus with metoprolol (ref. 297) and propranolol (ref. 298) blood levels were increased, whereas the kinetics of acebutolol and its major metabolite diacetolol (ref. 297), of timolol (ref. 206) and of pindolol (ref. 207) were unaffected. Plasma concentrations of oxprenolol were increased, without a significant alteration of its effect (ref. 299). Poor absorption of nadolol did not allow a firm conclusion to be drawn

regarding the effect of hydralazine (ref. 297). The antiarrhythmic drug propafenone increased the steady-state levels of metoprolol and decreased its oral clearance (ref. 300). Quinidine had no effect on propranolol disposition (ref. 301). Flecainid increased the AUC for propranolol but did not affect its terminal half-life (ref. 302), and prazosin did not affect the fate of timolol (ref. 206). There was no pharmacokinetic interaction between isosorbide dinitrate and atenolol or propranolol (ref. 303). Atropine reduced the absorption of metipranolol (ref. 120).

Of the drugs used in complex antiulcer therapy, propantheline prolonged the absorption phase of atenolol (ref. 304) and decreased the C_{max} of metoprolol with prolongation of t_{max} (ref. 166). Metoclopramide did not affect the kinetics of atenolol (ref. 304), but caused an increase in the C_{max} of metoprolol (ref. 166). Cimetidine caused no significant changes in the kinetics of bisoprolol (ref. 305), betaxolol (ref. 306), atenolol (refs. 307-310), pindolol (ref. 307) or penbutolol (ref. 311), but it increased the blood levels of metoprolol (refs. 308-310, 312), labetalol (ref. 313) and propranolol (refs. 306, 310, 314-320) through decreased oral clearance. Ranitidine had no influence on the kinetics of propranolol (refs. 321-323) and atenolol (ref. 324), but increased the blood levels of metoprolol (refs. 324, 325). This interaction appears to be without clinical relevance because of the lack of pharmacodynamic interaction (ref. 325). It can be seen that for beta-blockers only partly biotransformed by the liver the inhibition of the oxidative metabolism by cimetidine has no appreciable influence on their overall metabolism, whereas the metabolic interaction of cimetidine increases the systemic bioavailability of drugs which undergo first-pass elimination (ref. 307). Ranitidine, on the other hand, had a negligible influence on the kinetics of propranolol and its interaction with metoprolol is without clinical relevance. Administration of antacid increased the plasma level of metoprolol, whereas for atenolol the opposite (ref. 304) or no effect (ref. 326) was observed. With propranolol the bioavailability was reduced when it was taken with aluminium hydroxide (ref. 77). Sucralfate significantly decreased the absorption of indenolol (ref. 328).

Diuretics are frequently co-administered with beta-blockers. The results indicating no interactions are summarized in Table 2.1.

TABLE 2.1

Diuretic	Beta-blocker	Reference
Chlorthalidone	Metoprolol	119
	Atenolol	329-331
Hydrochlorthiazide	Metoprolol	332, 333
	Acebutolol	335
	Pindolol	207
Furosemide	Atenolol	326
Bendrofluazide	Propranolol	336
Clopamide	Pindolol	208
Triamterene	Propranolol	334

Furosemide, however was reported to increase propranolol plasma levels (ref. 337).

For drugs acting on the central nervous system the following interactions were reported. The kinetics of neither atenolol or metoprolol were influenced by the concurrent administration of amitryptyline (ref. 338). The oral bioavailability of alprenolol (ref. 339) and metoprolol (ref. 340) was decreased by pentobarbital and that of timolol by phenobarbital (ref. 206). Alcohol intake increased the clearance of propranolol, which is metabolised in the liver, whereas that of sotalol, which is eliminated unchanged, was reduced (ref. 256). In another study, ethanol had no effect on the kinetics of acebutolol (ref. 205). Chlorpromazine reduced the oral clearance of propranolol (ref. 341).

From the available studies on chemotherapeutics it is known that coadministration of rifampicin resulted in a decrease in blood levels of propranolol (ref. 342), bisoprolol (ref. 305) and metoprolol (ref. 343). After administration of ampicillin the bioavailability of atenolol was reduced (ref. 344).

Of the group of analgesics and antiinflammatory agents, piroxicam did not alter the kinetics of atenolol and metoprolol (ref. 345), allopurinol and aspirin did not alter the kinetics of atenolol (ref. 344) and aspirin that of pindolol (ref. 207).

Calcium altered the kinetics of atenolol: C_{max} decreased and $t_{1/2}$ increased (ref. 326). Activated charcoal administered 3 h after an oral dose of nadolol led to a decrease in AUC, recovery of nadolol in the urine and its $t_{1/2}$ (ref. 346). When activated charcoal was given within 5 min of sotalol administration, its absorption was reduced by 99% (ref. 347).

In young women the plasma AUC of metoprolol was greater for

the pill group (ref. 348), whereas for oxprenolol, propranolol and atenolol the changes were not significant (ref. 349).

Finally, in smokers the clearance of propranolol was greater than that in non-smokers (refs. 218-220, 350, 351). With metoprolol the only significant difference between smokers and non-smokers was that smokers had a larger steady-state distribution volume (3.3 vs. 2.5 l/kg). There were no differences in half-life, systemic clearance or bioavailability (ref. 352).

2.18 PHARMACOKINETICS OF SLOW-RELEASE FORMULATIONS

In long-term treatment with beta-blockers compliance of the patient with the physician's prescription is essential for the success of therapy. The daily dose can be administered once per day or divided into two equal doses twice per day. The once per day administration is accompanied by a greater difference between peak and trough levels in comparison with the twice per day mode, although the steady state AUC from time 0 to 24 h may be equal for both regimens. The great variation of the blood levels in the once daily regimen may be accompanied by undesirable side-effects. On the other hand, compliance can be improved by restricting the number of daily doses. Taking these facts into account, for several beta-blockers forms were developed with controlled release of the effective substance. The pharmacokinetics of several beta-blockers after administration of such drug forms will be briefly characterized in the following section.

2.18.1 Metoprolol

The in vitro dissolution rate of metoprolol controlled-release preparation based on the matrix principle (Durules) was considerably lower than that of conventional metoprolol tablets. Nevertheless, the preparations of the matrix type release about 80-100% of the dose within 8-10 h. This means that absorption occurs only during the first half of the dosage interval when the products are administered once daily. Correspondingly, the maximum concentration of metoprolol in plasma after taking Durules (0.2 g) was about half of that after two regular tablets (2X0.1 g). A lower extent of bioavailability was observed for Durules than for the ordinary tablets. This higher first-pass effect for slowly absorbed metoprolol can be explained by a reciprocal relationship between the uptake by the liver and the drug concentration in the portal venous blood (ref. 353). Once daily treatment

of hypertension with metoprolol, even in a "long-acting" formulation of the matrix type, was not recommended because of the waning antihypertensive effect which would be missed on routine clinic attendance (ref. 354).

The osmotic drug delivery system (OROS), providing release of the drug with zero-order kinetics, led to a further delay in time to peak and constant plasma levels over longer periods compared with the commercial matrix-type slow-release formulation. The same total amount of drug reached the circulation from the two formulations, as shown by the similarity of the AUC values (ref. 355).

Another approach is to divide the dose into several small units such as coated pellets (multiple unit system). In vitro/in vivo correlation studies with such a "multiple-unit" metoprolol tablet showed a pH-independent and constant (zero-order) release in the course of about 20 h. The extended absorption process resulted in a uniform plasma concentration curve without marked peaks and troughs, as indicated by a C_{max}/C_{min} ratio of 1.8 $\pm$ 1.3 (mean $\pm$ S.D.). When the same daily dose was given to the subjects as conventional tablets every 12 h the corresponding ratio was 8.4 $\pm$ 6.2 (ref. 356).

2.18.2 Propranolol

The long-acting product consists of a hard gelatin capsule filled with a multitude of small spherules of propranolol hydrochloride individually coated with a semipermeable membrane to allow diffusion release of the drug in a controlled manner. In vitro dissolution studies have indicated that the time taken for 90% of the dose to dissolve from one of the long-acting capsules is in excess of 12 h (ref. 357). The peak propranolol blood level for the long-acting formulation was lower than that obtained with the standard tablet. However, from about 12 h on the levels for the long-acting formulation were higher (refs. 357-360). The areas under the curves are significantly reduced for the long-acting formulation in comparison with those achieved by conventional propranolol in single or divided doses (refs. 357, 358, 361, 362). This difference could be accounted for by a higher degree of metabolism on the first pass through the liver due to a slower rate of absorption (refs. 357, 360, 363). The long-acting formulation is shown to have an apparent half-life of 12.7 h, but this is probably due to a combination of absorption and elimination proceeding simultaneously for an extended time (ref. 357). In another study

on the pharmacokinetics of sustained-release propranolol, the half-lives of the three different formulations were inversely proportional to their dissolution rates (ref. 360).

For details of the pharmacokinetics of long-acting propranolol, see the review by Nace and Wood (ref. 364).

2.18.3 Oxprenolol

From the matrix-type slow-release tablet 100% of the substance was released within 6 h in comparison with 2 h for the standard rapid-release formulation. Oxprenolol peak plasma concentrations were lower with slow-release than with rapid-release tablets and the peak was delayed. The relative bioavailabilities of the two formulations were similar (ref. 365). This is in contrast to the slow-release preparations of some other beta-blockers, where the apparent bioavailability relative to the rapid-release preparation was reduced (see Sections 2.18.1 and 2.18.2), and for alprenolol (ref. 366). The osmotic delivery system (OROS) performed reproducibly and maintained plasma concentrations over 24 h sufficient to provide significant levels of beta-adrenoceptor blockade (ref. 367). Variations in mean plasma levels and beta-adrenoceptor blockade were considerably reduced on the OROS once-daily regimen compared with the polymer-matrix oxprenolol hydrochloride twice--daily regimen (ref. 368).

2.18.4 Metipranolol

A pharmacokinetic comparison of the slow-release preparation of metipranolol with the conventional tablet showed bioequivalence of both formulations and a prolongation of the elimination half-life for the slow-release dosage form (ref. 369).

REFERENCES

1 W. Riess, S. Brechbühler, L. Brunner, P.R. Imhoff and D.B. Jack, in W. Schweizer (Editor), Beta-blockers - present status and future prospects, Hubert, Bern, Stuttgart, Vienna, 1974, pp. 276-289.
2 D.G. Shand, Drugs, 7 (1974) 39-47.
3 A.S. Nies and D.G. Shand, Circulation, 5 (1975) 6-15.
4 S. Pfeifer and C. Zimmer, Pharmazie, 30 (1975) 625-633.
5 C.G. Regårdh, Acta Pharmacol. Toxicol., 37 (suppl. 1) (1975) 1-39.
6 G. Johnsson and C.G. Regårdh, Drugs, 11 (suppl. 1) (1976) 111--121.

62

7   G. Johnsson and C.G. Regårdh, Clin. Pharmacokinet., 1 (1976)
    233–263.
8   D.G. Shand, Postgrad. Med. J., 52 (suppl. 4) (1976) 22–25.
9   J. Meier, Curr. Med. Res. Opin., 4 (suppl. 5) (1977) 31–38.
10 J.R. Kiechel and J. Meier, Nouv. Presse Méd., 7 (1978) 2685–
    2687.
11 J. Meier, Cardiology, 64 (1979) 1–13.
12 R. Gugler, R. Krist, H. Raczinski, K. Höffgen and G. Bodem,
    Br. J. Clin. Pharmacol., 10 (1980) 337–343.
13 P.A. Routledge and D.G. Shand, in W.E. Evans, J.J. Schentag and
    W.J. Jusko (Editors), Applied Pharmacokinetics (Principles of
    Therapeutic Drug Monitoring), Applied Therapeutics, San Fran-
    cisco, 1980, pp. 464–485.
14 A.J. Smith and G.T. Tucker, in L. Szekeres (Editor), Handbook
    of Experimental Pharmacology, Springer-Verlag, Berlin, vol. 54
    (2),1980, pp. 417–504.
15 G.R. Bourne, in J.W. Bridges and L.F. Chasseaud (Editors),
    Progress in Drug Metabolism, John Wiley and Sons, Ltd., vol. 6,
    1981, pp. 77–108.
16 C.G.D. Cowling and W.P. Leary, Curr. Ther. Res., 30 (1981)
    765–774.
17 L.K. Golightly, Pharmacotherapy, 2 (1982) 134–147.
18 E.P. MacCarthy and S.S. Bloomfield, Pharmacotherapy, 3 (1983)
    193–219.
19 A.J.J. Wood and J. Feely, Clin. Pharmacokinet., 8 (1983) 253–
    262.
20 M.J. Boutroy, Dev. Pharmacol. Ther., 10 (1987) 224–231.
21 S. Nattel, G. Gagne and M. Pineau, Clin. Pharmacokinet., 13
    (1987) 293–316.
22 J.G. Riddell, D.W.G. Harron and R.G. Shanks, Clin. Pharmaco-
    kinet., 12 (1987) 305–320.
23 P.H. Hinderling, O. Schmidlin and J.K. Seydel, J. Pharmacokin.
    Biopharm., 12 (1984) 263–287.
24 M. Esler, A. Zweifler, O. Randall and V. DeQuattro, Clin.
    Pharmacol. Ther., 22 (1977) 299–308.
25 K.L. Woods, S.P. Linton, M.J. Kendall, E.B. Faragher and R.J.
    Grieve, Eur. J. Clin Pharmacol., 15 (1979) 229–233.
26 J.F. Mullane, J. Kaufman, D. Dvornik and J. Coelho, Clin. Phar-
    macol. Ther., 32 (1982) 692–700.
27 K.P. Öhman, J. Asplund, S. Landahl and B. Liander, Eur. J.
    Clin. Pharmacol., 22 (1982) 95–99.
28 C. Davidson, V. Thadani, S.H. Taylor, H. Hess and W. Riess,
    Eur. J. Clin. Pharmacol., 10 (1976) 189–195.
29 M.J. Kendall and V.A. John, Am. J. Cardiol., 52 (1983) 27D–33D.
30 O. Rönn, E. Fellenius, C. Graffner, G. Johnsson, P. Lundborg
    and L. Svensson, Eur. J. Clin. Pharmacol., 17 (1980) 81–86.
31 T. Ishizaki, A. Ohnishi, T. Sasaki, K. Chiba, T. Suganuma and
    K. Kushida, Eur. J. Clin. Pharmacol., 25 (1983) 749–757.
32 B. Åblad, M. Ervik, J. Hallgren, G. Johnsson and L. Sölvell,
    Eur. J. Clin. Pharmacol., 5 (1972) 44–52.
33 B. Åblad, K.O. Borg, G. Johnsson, G. Regårdh and L. Sölvell,
    Life Sci., 14 (1974) 693–704.
34 R. Gugler, W. Höbel, G. Bodem and H.J. Dengler, Clin. Pharma-
    col. Ther., 17 (1975) 127–133.
35 W.H. Aellig, Arch. Int. Pharmacodyn. Thér., Suppl. (1980) 32–
    37.
36 J.B. Kostis, C.R. Lacy, S.D. Krieger and N.M. Cosgrove, Amer.
    Heart J., 108 (1984) 1131–1136.
37 D.J. Coltart and D.G. Shand, Br. Med. J., 3 (1970) 731–734.

38 C.A. Chidsey, P. Morselli, G. Bianchetti, A. Morganti, G. Leo-
   netti and A. Zanchetti, Circulation, 52 (1975) 313-318.
39 T. Ishizaki and K. Tawara, Eur. J. Clin. Pharmacol., 14 (1978)
   7-14.
40 C.G. Regårdh, G. Johnsson, L. Jordö, P. Lundborg, B.A. Persson
   and O. Rönn, J. Cardiovasc. Pharmacol., 2 (1980) 715-723.
41 A. Ebihara, K. Tawara, T. Oka, T. Ofuji and K. Kawahara, Eur.
   J. Clin. Pharmacol., 23 (1982) 189-195.
42 K. Tawara, E. Steiner and C. von Bahr, Eur. J. Clin. Pharma-
   col., 31 (1987) 667-672.
43 H.C. Brown, S.G. Carruthers, G.D. Johnston, J.G. Kelly, J.
   McAinsh, D.C. McDevitt and R.G. Shanks, Clin. Pharmacol.
   Ther., 20 (1976) 524-534.
44 A. Amery, J.F. De Plaen, P. Lijnen, J. McAinsh and T.
   Reybrouck, Clin. Pharmacol. Ther., 21 (1977) 691-699.
45 J. McAinsh, Postgrad. Med. J., 53 (Suppl. 3) (1977) 74-78.
46 J.K. Faulkner, D.A. Stopher, R. Walden, W. Singleton and S.H.
   Taylor, Br. J. Clin. Pharmacol., 2 (1975) 423-428.
47 H.C. Brown, S.G. Carruthers, J. G. Kelly, D.G. McDevitt and
   R.G. Shanks, Eur. J. Clin. Pharmacol., 9 (1976) 367-372.
48 S.G. Carruthers, P. Pentikainen, J.P. Hosler and D.L. Azarnoff,
   Clin. Pharmacol. Ther., 26 (1979) 682-685.
49 S.G. Carruthers, J.G. Kelly, D.G. McDevitt and R.G. Shanks,
   Clin. Pharmacol. Ther., 15 (1974) 497-509.
50 C.Y. Sum, A. Yacobi, R. Kartzinel, H. Stampfli, C.S. Davis and
   C.M. Lai, Clin. Pharmacol. Ther., 34 (1983) 427-434.
51 M. Eckert, G. Cocco, C. Strozzi, P. Heizmann and C. Sfrisi,
   Eur. J. Clin. Pharmacol., 24 (1983) 479-484.
52 R.K. Ferguson, P.H. Vlasses, J.R. Koplin, G.I. Holmes, P.
   Huber, J. Demetriades and W.B. Abrams, Br. J. Clin. Pharmacol.,
   14 (1982) 719-725.
53 G. Levy, Clin. Pharmacol. Ther., 7 (1966) 362-372.
54 M. Gibaldi and D. Perrier, Pharmacokinetics, Marcel Dekker,
   Inc., New York, 1982.
55 T. Ishizaki, Res. Comm. Chem. Pathol. Pharmacol., 27 (1980)
   223-239.
56 W.A. Ritschel and A. Hussain, Meth. Find. Exptl. Clin.
   Pharmacol., 6 (1984) 627-640.
57 N.H.G. Holford and L.B. Sheiner, Pharmac. Ther., 16 (1982) 143-
   166.
58 C.R. Cleaveland and D.G. Shand, Clin. Pharmacol. Ther., 13
   (1972) 181-185.
59 M.T. Smith, I. Livingstone, M.J. Eadie, W.D. Hooper and E.J.
   Triggs, Eur. J. Clin. Pharmacol., 25 (1983) 481-490.
60 T. Reybrouck, A. Amery, R. Fagard, P. Jonsten, P. Lijmen and E.
   E. Meulepas, Br. Med. J., 1 (1978) 1386-1388.
61 M. Chaignon, M. Lucsko, P. Aubert and J. Guédon, Arch. Mal.
   Coeur, 75 (1982) 963-969.
62 M.A. Martin, F.C. Phillips, G.T. Tucker and A.J. Smith, Eur. J.
   Clin. Pharmacol., 14 (1978) 383-390.
63 S.N. Anavekar, W.J. Louis, T.O. Morgan, A.E. Doyle and C.I.
   Johnston, Clin. Exp. Pharmacol. Physiol., 2 (1975) 203-212.
64 R. Platzer, R.L. Galeazzi. W. Niederberger and J. Rosenthaler,
   Clin. Pharmacol. Ther., 36 (1984) 5-13.
65 A. Wellstein, D. Palm, G.G. Belz and H.F. Pitschner, Arzneim.-
   Forsch., 35 (1985) 2-6.
66 J.G. Wagner, J. Theoret. Biol., 20 (1968) 173-201.
67 A. Wellstein, D. Palm, H.F. Pitschner and G.G. Belz, Eur. J.
   Clin. Pharmacol., 29 (1985) 131-147.

68 A. Wellstein and D. Palm, Eur. J. Clin. Pharmacol., 29 (1985) 293-300.
69 A. Wellstein, D. Palm, G.G. Belz, G. Leopold, K.V. Bühring and J. Pabst, J. Cardiovasc. Pharmacol., 8 (suppl. 11) (1986) 41S-45S.
70 J.G. Wagner, Fundamentals of clinical pharmacokinetics,Drug Intelligence Publications, Inc., Hamilton, Ill., USA, 1975.
71 L. Brunner, P. Imhof and D. Jack, Eur. J. Clin. Pharmacol., 8, (1975) 3-9.
72 W.D. Mason and N. Winer, Clin. Pharmacol. Ther., 20 (1976) 401-412.
73 B. Silke, V.A. John, R.T. Calvert and S.H. Taylor, Eur. J. Clin. Pharmacol., 24 (1983) 7-14.
74 V. Abshagen, G. Betzien, B. Kaufmann and G. Endele, Eur. J. Clin. Pharmacol., 21 (1982) 293-301.
75 M.A. Klausner, J.B. Coelho, D. Dvornik, C.A. Perdomo, D.G. Shand, D.J. Weidler and J.F. Mullane, Curr. Ther. Res., 36 (1984) 379-387.
76 R. Gugler and G. Bodem, Eur. J. Clin. Pharmacol., 13 (1978) 13-16.
77 G.L. Jennings, A. Bobik, E.T. Fagan and P.I. Korner, Br. J. Clin. Pharmacol., 7 (1979) 245-256.
78 J.J. Lima, P.F. Binkley, J. Johnson and C.V. Leier, J. Clin. Pharmacol., 26 (1986) 253-257.
79 K.D. Haegele, P. Jaillon, G. Cheymol, R.G. Alken, P.J. Schechter and J. Koch-Weser, Clin. Pharmacol. Ther., 34 (1983) 785-791.
80 M. Schäfer-Korting, N. Bach, H. Knauf and E. Mutschler, Eur. J. Clin. Pharmacol., 26 (1984) 125-127.
81 J.R. Latts, Angiology, (1986) 221-225.
82 R. Sega, F. Mailland, F. Colombo, G. Groothold, C. Pantarotto, G. Guenzati and A. Libretti, J. Clin. Pharmacol., 25 (1985) 337-342.
83 P.J. Meffin, R.A. Winkle, F.A. Peters and D.C. Harrison, Clin. Pharmacol. Ther., 22 (1977) 557-567.
84 G. Johnsson, C.G. Regårdh and L. Sölvell, Acta Pharmacol. Toxicol. 36 (suppl. 5) (1975) 31-44.
85 P. Vermeij, M. El Sherbini-Schepers and P.A. Van Zwieten, J. Pharm. Pharmacol., 30 (1978) 53-55.
86 A. Yacobi, R. Kartzinel, C.M. Lai and C.Y. Sum, J. Pharm. Sci., 72 (1983) 710-711.
87 G. Hitzenberger, F. Takacs and H. Pittner, Arzneim.-Forsch., 33, (1983) 50-52.
88 R.J. Norris, E.H. Lee, D. Muirhead and S.W. Sanders, J. Cardiovasc. Pharmacol., 8 (suppl. 4) (1986) S91-S92.
89 G.H. Evans and D.G. Shand, Clin. Pharmacol. Ther., 14 (1973) 487-493.
90 R.G. McAllister, Clin. Pharmacol. Ther., 20 (1976) 517-523.
91 T. Hanssen, T. Heyden, I. Sundberg, G. Alfredsson, H. Nyback and L. Wetterberg, Arch. Gen. Psychiat., 37 (1980) 685-690.
92 B.M. Silber, N.H.G. Holford and S. Riegelman, J. Pharm. Sci., 72 (1983) 725-732.
93 Z. Kopitar, B. Vrhova, A. Lenardič, P. Cvelbar, M. Žorž and I. Francetič, Int. J. Clin. Pharmacol. Ther. Toxicol., 24 (1986) 319-322.
94 D.W. Schneck, J.F. Pritchard, T.P. Gibson, J.E. Vary and A.H. Hayes, Clin. Pharmacol. Ther., 27 (1980) 744-755.
95 J.G. Wagner, Clin. Pharmacol. Ther. 37 (1985) 481-487.
96 J. McAinsh and M.A. Gay, Eur. J. Drug Metab. Pharmacokin., 10 (1985) 241-245.

97 T. Walle, E.C. Conradi, U.K. Walle, T.C. Fagan and T.E. Gaffney, Clin. Pharmacol. Ther. 24 (1978) 668-677.
98 F. Albani, R. Riva, A. Baldrati, P. Cortelli, E. Perucca, G. Procaccianti and A. Baruzzi, Int. J. Clin. Pharm. Res., 4 (1984) 19-23.
99 B. Lemmer, in D.D. Breimer and P. Speiser (Editors), Topics in Pharmaceutical Sciences, Elsevier/North Holland, Amsterdam, New York, Oxford, 1981, pp. 46-68.
100 K. Semenowicz-Siuda, A. Markiewicz and J. Korczynska-Wardecka, Int. J. Clin. Pharmacol. Therap. Toxicol., 22 (1984) 653-658.
101 B. Lemmer and K. Bathe, J. Cardiovasc. Pharmacol., 4 (1982) 635-644.
102 B. Lemmer, K. Bathe, P.H. Lang, G. Neumann and H. Winkler, J. Amer. Coll. Toxicol., 2 (1983) 347-358.
103 B. Lemmer, H. Winkler, T. Ohm and M. Fink, Naunyn-Schmiedeberg's Arch. Pharmacol., 330 (1985) 42-49.
104 M. Rowland and T.N. Tozer, Clinical Pharmacokinetics, Lea and Fibiger (Editors), Philadelphia, 1980.
105 R.A. Branch, D.G. Shand, G.R. Wilkinson and A.S. Nies, J. Pharmacol. Exp. Ther., 184 (1973) 515-519.
106 M.J. Kendall and C.P. Quarterman, Int. J. Clin. Pharmacol. Ther. Toxicol., 20 (1982) 101-104.
107 M. Anttila, M. Arstila, M. Pfeffer, R. Tikkanen, U. Vallinkoski and H. Sundquist, Acta Pharmacol. Toxicol., 39 (1976) 118-128.
108 G. Bianchetti, C. Blatrix, R. Gomeni, J.R. Kilborn, J. Larribaud, P.W. Lucker, J.J. Thebault, S. Trocherie and P.L. Morselli, Arzneim.-Forsch., 30 (1980) 1912-1916.
109 J. Bonelli, G. Hitzenberger, W. Krause, H. Wendt and U. Speck, Int. J. Clin. Pharmacol. Ther. Toxicol., 18 (1980) 169-176.
110 R.L. Galeazzi, M. Pirovino and P. Weidmann, Clin. Pharmacol. Ther., 33 (1983) 733-740.
111 G. Maurer, P. Donatsch, H. Galliker, J.R. Kiechel and J. Meier, Xenobiotica, 11 (1981) 33-41.
112 N. Bernard, G. Cuisinaud, N. Poset, P.Y. Zech and J. Sassard, Eur. J. Clin. Pharmacol., 29 (1985) 215-219.
113 E. Riva, R. Latini, A. Cremonesi, F. Zaca and P. Pagliarani, J. Cardiovasc. Pharmacol., 2 (1980) 707-714.
114 A.J.J. Wood, J. Carr, R.E. Vestal, S. Belcher, G.R. Wilkinson and D.G. Shand, Br. J. Clin. Pharmacol., 6 (1978) 345-350.
115 R.J. Straka, R.L. Lalonde, J.A. Pieper, M.B. Bottorff and D.M. Mirvis, J. Pharm. Sci., 76 (1987) 521-524.
116 C. Bengtsson, G. Johnsson and C.G. Regårdh, Clin. Pharmacol. Ther., 17 (1975) 400-408.
117 M.J. Kendall, V.A. John, C.P. Quarterman and P.G. Welling, Eur. J. Clin. Pharmacol., 17 (1980) 87-92.
118 M.G. Myers and J.J. Thiessen, Clin. Pharmacol. Ther., 27 (1980) 756-762.
119 J. Godbillon, A. Gerardin, V.A. John and W. Theobald, Eur. J. Clin. Pharmacol., 24 (1983) 655-660.
120 O. Mayer, V. Čepelák, J. Vitouš and J. Potměsil, Int. J. Clin. Pharmacol. Ther. Toxicol., 18 (1980) 113-120.
121 M.S. Lennard, G.T. Tucker and H.F. Woods, Clin. Pharmacokin., 11 (1986) 1-17.
122 M.S. Lennard, J.H. Silas, S. Freestone, L.E. Ramsey, G.T. Tucker and H.F. Woods, New Engl. J. Med., 307 (1982) 1558-1560.
123 M.S. Lennard, J.H. Silas, S. Freestone and J. Trevethick, Br. J. Clin. Pharmacol., 14 (1982) 301-303.

124 M.S. Lennard, S. Freestone, L.E. Ramsay, G.T. Tucker and H.F. Woods, New Engl. J. Med., 308 (1983) 965-966.

125 D.A.P. Evans, A. Mahgoub, T.P. Sloan, J.R. Idle and R.L. Smith, J. Med. Genet., 17 (1980) 102-105.

126 M. Eichelbaum, Clin. Pharmacokinet., 7 (1982) 1-22.

127 M.S. Lennard, J.H. Silas, S. Freestone, G.T. Tucker, L.E. Ramsay and H.F. Woods, Br. J. Clin. Pharmacol., 16 (1983) 586P-587P.

128 J.H. Silas, M.S. Lennard, G.T. Tucker, L.E. Ramsay and H.F. Woods, Br. J. Clin. Pharmacol., 17 (1984) 11S-19S.

129 P. Dayer, L. Balant, F. Courvoisier, A. Kupfer, A. Kubli, A. Gorgia and J. Fabre, Eur. J. Drug Metab. Pharmacokinet., 7 (1982) 73-77.

130 P. Dayer, A. Kubli, F. Courvoisier, L. Balant and J. Fabre, Br. J. Clin. Pharmacol., 13 (1982) 750-752.

131 P. Dayer, F. Courvoisier, L. Balant and J. Fabre, Lancet, 1 (1982) 509.

132 P. Dayer, L. Balant, A. Kupfer, F. Courvoisier and J. Fabre, Eur. J. Clin. Pharmacol., 24 (1983) 797-799.

133 R.V. Lewis, M.S. Lennard, P.R. Jackson, G.T. Tucker, L.E. Ramsay and H.F. Woods, Br. J. Clin. Pharmacol., 19 (1985) 329-333.

134 P. Dayer, G. Mérier, J.J. Perrenoud, A. Marmy and T. Leemann, J. Cardiovasc. Pharmacol., 8 (suppl. 6) (1986) S20-S24.

135 M.S. Lennard, P.R. Jackson, F. Freestone, L.E. Ramsay, G.T. Tucker and H.F. Woods, Br. J. Clin. Pharmacol., 17 (1984) 106S-107S.

136 T.C. Raghuram, R.P. Koshakji, G.R. Wilkinson and A.J.J. Wood, Clin. Pharmacol. Therap., 36 (1984) 51-56.

137 M.S. Lennard, L.E. Ramsey, G.T. Tucker and H.F. Woods, Pharm. Int., 4 (1983) 61-65.

138 R.G. Cooper and D.A.P. Evans, Lancet, 2 (1984) 227.

139 J.H. Silas, J.C. McGourty, M.S. Lennard, G.T. Tucker and H.F. Woods, Eur. J. Clin. Pharmacol., 28 (suppl.) (1985) 85-88.

140 P. Dayer, T. Leemann, A. Marmy and J. Rosenthaler, Eur. J. Clin. Parmacol., 28 (1985) 149-153.

141 D.W.J. Clark, Drugs, 29 (1985) 342-375.

142 W. Kirch, E.E. Ohnhaus, C. Zekorn, M. Eichelbaum, H. Spahn and E. Mutschler, Arch. Toxicol., 8 (suppl.) (1985) 401-402.

143 J.C. McGourty, J.H. Silas, M.S. Lennard, G.T. Tucker and H.F. Woods, Br. J. Clin. Pharmacol., 20 (1985) 555-566.

144 P. Dayer, R. Gasser, J. Gut, T. Kronbach, G.M. Robertz, M. Eichelbaum and U.A. Meyer, Biochem. Biophys. Res. Commun., 125 (1984) 374-380.

145 J. Gut, R. Gasser, P. Dayer, T. Kronbach, T. Catin and U.A. Meyer, FEBS Lett., 173 (1984) 287-290.

146 T. Leemann, P. Dayer and U.A. Meyer, Eur. J. Clin. Pharmacol., 29 (1986) 739-741.

147 P. Dayer, L. Balant, A. Kupfer, R. Striberni and T. Leemann, Eur. J. Clin. Pharmacol., 28 (1985) 317-320.

148 P. Dayer, T. Leemann, A. Kupfer, T. Kronbach and U.A. Meyer, Eur. J. Clin. Pharmacol., 31 (1986) 313.318.

149 J.C. McGourty, J.H. Silas, J.J. Fleming, A. McBurney and J.W. Ward, Clin. Pharmacol. Ther., 38 (1985) 409-413.

150 E.J. Ariens, Eur. J. Clin. Pharmacol., 26 (1984) 663-668.

151 C.F. George, T. Fenyvesi, M.E. Conolly and C.T. Dollery, Eur. J. Clin. Pharmacol., 4 (1972) 74-76.

152 J. Hermansson and C. von Bahr, J. Chromatogr., 221 (1980) 109-117.

153 B. Silber and S. Riegelman, J. Pharmacol. Exp. Ther., 215 (1980) 643-649.
154 B. Silber, N.H.G. Holford and S. Riegelman, J. Pharm. Sci., 71 (1982) 699-703.
155 C. von Bahr and J. Hermansson and K. Tawara, Br. J. Clin. Pharmacol., 14 (1982) 79-82.
156 L.S. Olanoff, T. Walle, U.K. Walle, T.D. Cowart and T.E. Gaffney, Clin. Pharmacol. Ther., 35 (1984) 755-761:
157 T. Walle, Drug Metab. Dispos., 13 (1985) 279-282.
158 J. Hermansson and C. von Bahr, J. Chromatogr., 227 (1982) 113-127.
159 R.J. Francis, P.G. East and J. Larman, Eur. J. Clin. Pharmacol., 23 (1982) 529-533.
160 C. Harvengt and J.P. Desager, Int. J. Clin. Pharmacol. Ther. Toxicol., 20 (1982) 57-61.
161 P.H. Hsyu and K.M. Giacomini, J. Clin. Invest., 76 (1985) 1720-1726.
162 M.G. Sankey, A. Gulaid and C.M. Kaye, J. Pharm. Pharmacol., 36 (1984) 276-277.
163 U.K. Walle, T. Walle, S.A. Bai and L.S. Olanoff, Clin. Pharmacol. Ther., 34 (1983) 718-723.
164 J.D. Fitzgerald, R. Ruffin, K.G. Smedstad, R. Roberts and J. McAinsh, Eur. J. Clin. Pharmacol., 13 (1978) 81-89.
165 M.G. Myers, G.R.J. Lewis, J. Steiner and C.T. Dollery, Clin. Pharmacol. Ther., 19 (1976) 502-507.
166 R.H. Briant, R.E. Dorrington, D.G. Ferry and J.W. Paxton, Eur. J. Clin. Pharmacol., 25 (1983) 353-356.
167 D.B. Jack, C.P. Quarterman, R. Zaman and M.J. Kendall, Eur. J. Clin. Pharmacol., 23 (1982) 37-42.
168 N.P. DeBruijn, J.G. Reves, N. Croughwell, F. Clements and D.A. Drissel, Anestesiology, 66 (1987) 323-326.
169 R. Gugler, W. Herold and H.J. Dengler, Eur. J. Clin. Pharmacol., 7 (1974) 17-24.
170 M.J. Kendall, C.P. Quarterman, H. Bishop and R.E. Schneider, Br. Med. J., 2 (1979) 465-468.
171 M.D. Rawlins, P. Collste, M. Frisk-Holmberg, M. Lind, J. Östman and F. Sjöquist, Eur. J. Clin. Pharmacol., 7 (1974) 353-356.
172 E. Vervloet, B.F.M. Pluym, J. Cillssen, K. Köhlen and W.H.M. Merkus, Clin. Pharmacol. Ther., 22 (1977) 853-857.
173 L.D. DeLeve, L. Endrényi and F.H.H. Leenen, J. Clin. Pharmacol., 25 (1985) 182-186.
174 K.J. Thorley, J. McAinsh and J.M. Cruickshank, Br. J. Clin. Pharmacol., 12 (1981) 725-730.
175 K.J. Thorley and J. McAinsh, Biopharm. Drug. Dispos., 4 (1983) 299-301.
176 L. Nylund, N.O. Lunell, R. Lewander, B. Sarby and S. Thornström, Arch. Obstet. Gynaecol. Scand., 118 (suppl.) (1984) 71-73.
177 P.C. Rubin, L. Butters, A.N. Kelman, C. Fitzsimons and J.L. Reid, Br. J. Clin. Pharmacol., 15 (1983) 465-470.
178 S. Högstedt, B. Lindberg, D.R. Peng, C.G. Regårdh and A. Rane, Clin. Pharmacol. Ther., 37 (1985) 688-692.
179 S. Lindeberg, P. Lundborg, C.G. Regårdh and B. Sandström, Eur. J. Clin. Pharmacol., 33 (1987) 363-368.
180 J.T. Wilson, R.D. Brown, D.R. Cherek, J.W. Dailey, B. Hilman, P.C. Jobe, B.R. Manno, J.E. Manno, H.M. Redetzki and J.J. Stewart, Clin. Pharmacokinet., 5 (1980) 1-66.
181 F. Rassmusen, in C. Galli, G. Jacini and A. Peeile (Editors), Dietary lipids and postnatal development, Raven Press, New

York, 1973, pp. 231-245.

182 M. Liedholm, A. Melander, P.O. Bitzén, G. Helm, G. Lönnerholm, I. Mattiasson, B. Nilsson and E. Wåhlin-Boll, Eur. J. Clin. Pharmacol., 20 (1981) 229-231.

183 J. Kulas, N.O. Lunell, U. Rosing, B. Stéen and A. Rane, Acta Obstet. Gynaecol. Scand., 118 (suppl.) (1984) 65-69.

184 S. Lindeberg, B. Sandström, P. Lundborg and C.G. Regårdh, Acta Obstet. Gynaecol. Scand., 118 (suppl.) (1984) 61-64.

185 R.K. Bhamra, K.J. Thorby, J.A. Vale and D.W. Holt, Therap. Drug Monit., 5 (1983) 313-318.

186 S.H.Y. Wong, Clin. Lab. Sci., 15 (1985) 100-105.

187 J. Fidler and V. Smith, Br. J. Obstet. Gynaecol., 90 (1983) 961-965.

188 W. Krause, I. Stopelli, S. Milia and E. Rainer, Eur. J. Clin. Pharacol., 22 (1982) 53-55.

189 B. Karlberg, D. Lundberg and H. Alberg, Acta Pharmacol. Toxicol., 34 (1974) 222-224.

190 A.A. Levitan and J.C. Manion, Am. J. Cardiol., 32 (1973) 247.

191 J.H. Bauer, B. Pape, I. Zajicek and T. Grosborg, Am. J. Cardiol., 43 (1979) 860-862.

192 A. Melander, K. Danielson, B. Schersten and E. Wahlin, Clin. Pharmacol. Ther., 22 (1977) 108-112.

193 A. Melander, P. Stenberg, H. Liedholm, B. Scherstén and E. Wåhlin-Boll, Eur. J. Clin. Pharmacol., 16 (1979) 327-330.

194 A.J. McLean, P.J. McNamara, P. du Souich, M. Gibaldi and D. Lalka, Clin. Pharmacol. Ther., 24 (1978) 5-10.

195 A.J. McLean, C. Isbister, A. Bobik and F.J. Dudley, Clin. Pharmacol. Ther., 30 (1981) 31-34.

196 T. Walle, T.C. Fagan, U.K. Walle, M.J. Oexmann, E.C. Conradi and T.E. Gaffney, Clin. Pharmacol. Ther., 30 (1981) 790-795.

197 L.S. Olanoff, T. Walle, T.D. Cowart, U.K. Walle, M.J. Oexmann and E.C. Conradi, Clin. Pharmacol. Therap., 40 (1986) 408-414.

198 R. Mäntylä, H. Allonen, J. Kanto, T. Kleimola and R. Sellman, Br. J. Clin. Pharmacol., 9 (1980) 435-437.

199 T.K. Daneshmend and C.J.C. Roberts, Br. J. Clin. Pharmacol., 14 (1982) 73-78.

200 T.C. Fagan, T. Walle, M.J. Oexman, U.K. Walle, S.A. Bai and T.E. Gaffney, Clin. Pharmacol. Ther., 41 (1987) 402-406.

201 J. Feely, J. Nadeau and A.J.J. Wood, Br. J. Clin. Pharmacol., 15 (1983) 383-385.

202 A.J. Byrne, J.J. McNeil, P.M. Harrison, W. Louis, A.M. Tonkin and A.J. McLean, Br. J. Clin. Pharmacol., 17 (1984) 45S-50S.

203 C.P. Dawes, M.J. Kendall and P.G. Welling, Br. J. Clin. Pharmacol., 7 (1979) 299-302.

204 G. Leopold, J. Pabst, W. Ungethum and K.U. Buhring, J. Clin. Pharmacol., 26 (1986) 616-621.

205 R. Zaman, M.R. Wilkins, M.J. Kendall and D.B. Jack, Biopharm. Drug Dispos., 5 (1984) 91-95.

206 R. Mäntylä, P. Männisto, S. Nykänen, A. Koponen and U. Lamminsivu, Eur. J. Clin. Pharmacol., 24 (1983) 227-230.

207 H.J. Schwarz, Amer. Heart J., 104 (1982) 357-364.

208 J.L. Kiger, D. Lavene, M.F. Guillaume, M. Guerret and J. Longchampt, Int. J. Clin. Pharmacol., 13 (1976) 228-232.

209 J. Godbillon, N. Vidon, R. Palma, A. Pfeiffer, C. Franchisseur, M. Bovet, G. Gosset, J.J. Bernier and J. Hirtz, Br. J. Clin. Pharmacol., 24 (1987) 335-341.

210 N. Vidon, D. Evard, J. Godbillon, M. Rongier, M. Duval, J.P. Schoeller, J.J. Bernier and J. Hirtz, Br. J. Clin. Pharmacol., 19 (1985) 107S-112S.

211 P. Kahela, M. Anttila, R. Tikkanen and H. Sundquist, Acta
    Pharmacol. Toxicol., 44 (1979) 7-12.
212 P. Lundborg and B. Steen, Acta Med. Scand., 200 (1976) 397-
    402.
213 M.J. Kendall, D. Brown and R.A. Yates, Br. J. Clin. Phar-
    macol., 4 (1977) 497-499.
214 C.P. Quarterman, M.J. Kendall and D.B. Jack, Br. J. Clin.
    Pharmacol., 11 (1981) 287-294.
215 C.G. Regårdh, S. Landahl, M. Larsson, P. Lundborg, B. Steen,
    K.J. Hoffmann and P.O., Lagerström, Eur. J. Clin. Pharmacol.,
    24 (1983) 221-226.
216 C.M. Castleden, C.M. Kaye and R.L. Parsons, Br. J. Clin.
    Pharmacol., 2 (1975) 303-306.
217 C.M. Castleden and C.F. George, Br. J. Clin. Pharmacol., 7
    (1979) 49-54.
218 J. Feely, J. Crooks and I.H. Stevenson, Br. J. Clin. Pharma-
    col., 12 (1981) 73-78.
219 T. Walle, R.P. Byington, C.D. Furberg, K.M. McIntyre and P.S.
    Vokonas, Clin. Pharmacol. Ther., 38 (1985) 509-518.
220 R.E. Vestal, A.J.J. Wood, R.A. Branch, D.G. Shand and G.R.
    Wilkinson, Clin. Pharmacol. Ther., 26 (1979) 8-15.
221 R.E. Schneider, H. Bishop, R.A. Yates, C.P. Quarterman and M.
    Kendall, Br. J. Clin. Pharmacol., 10 (1980) 169-170.
222 T. Ishizaki, H. Hirayama, K. Tawara, H. Nakaya, M. Sato and K.
    Sato, J. Pharmacol. Exp. Ther., 212 (1980) 173-181.
223 H. Spahn, W. Mühlberg, E. Mutschler and D. Platt, in D. Platt
    (Editor), Drugs and Ageing, Springer-Verlag, Berlin, Heidel-
    berg, 1986, pp. 164-170.
224 A. Roux, J.F. Henry, Y. Fonache, N.P. Chau, M.P. Hervy, F.
    Forrette, J.P. Bourdarias and B. Flouvat, Gerontology, 29
    (1983) 202-208.
225 W. Möhrke. E. Mutschler, W. Mühlberg and D. Platt, in D. Platt
    (Editor), Drugs and Ageing, Springer-Verlag, Berlin, Heidel-
    berg, 1986, pp. 144-151.
226 D.C. Brater and P. Chennavasin, in L.Z. Benet, N. Massoud and
    J.G. Gambertoglio (Editors), Pharmacokinetic Basis for Drug
    Treatment, Raven Press, New York, 1983, pp. 119-147.
227 J. Sassard, N. Pozet, J. McAinsh, J. Legheand and P. Zech,
    Eur. J. Clin. Pharmacol., 12 (1977) 175-180.
228 J. McAinsh, B.F. Holmes, S. Smith, D. Hood and D. Warren,
    Clin. Pharmacol. Ther., 28 (1980) 302-309.
229 W. Kirch, H. Köhler, E. Mutschler and M. Schäfer, Eur. J.
    Clin. Pharmacol., 19 (1981) 65-71.
230 T. Ishizaki, A. Ohnishi, T. Sasaki, K. Kushida, Y. Horai, K.
    Chiba and T. Suganuma, Eur. J. Clin. Pharmacol., 25 (1983) 95-
    101.
231 G. Hasenfuss, M. Schäfer-Korting, H. Knauf, E. Mutschler and
    H. Just, Eur. J. Clin. Pharmacol., 29 (1985) 461-465.
232 G. Bodem and C.A. Chidsey, Clin. Pharmacol. Ther., 14 (1973)
    26-29.
233 C.M. Kaye, C.R. Kumana, D.A. Franklin and L.R.I. Baker, Int.
    J. Clin. Pharmacol., 12 (1975) 83-88.
234 H.K. Sundquist, M. Anttila, J. Forsström and A. Kasanen, Ann.
    Clin. Res., 7 (1975) 442-446.
235 T.B. Tjandramaga, J. Thomas, R. Verbeeck, R. Verbesselt, R.
    Verberckmoes and P.J. De Schepper, Br. J. Clin. Pharmacol., 3
    (1976) 259-265.
236 J. Herrera, R.A. Vukovich and D.L. Griffith, Br. J. Clin.
    Pharmacol., 7 (suppl. 2) (1979) 227S-231S.

237 D.T. Lowenthal, J.M. Pitone, M.B. Affrime, J. Shirk, P. Busby, K.E. Kim, J. Nancarrow, C.D. Schwartz and G. Onesti, Clin. Pharmacol. Ther., 23 (1978) 606-615.
238 R. El-Rashidy, Biopharm Drug Disp., 2 (1981) 197-202.
239 W. Krause, D. Kampf and H.C. Fischer, Eur. J. Clin. Pharmacol., 27 (1984) 429-433.
240 L. Jordö, P.O. Attman, M. Aurell, L. Johansson, G. Johnsson and C.G. Regårdh, Clin. Pharmacokinet., 5 (1980) 169-180.
241 W. Tschöpe, J. Volk, U. Endele, U. Abshagen and E. Ritz, Verh. dtsch. Ges. Inn. Med., 85 (1979) 1226-1230.
242 A.J.J. Wood, R.E. Vestal, C.L. Spannuth, W.J. Stone, G.R. Wilkinson and D.G. Shand, Br. J. Clin. Pharmacol., 11 (1980) 561-566.
243 N.P. Chau, Y.A. Weiss, M.E. Safar, D.E. Lavene, D.R. Georges and P.L. Milliez, Clin. Pharmacol. Ther., 22 (1977) 505-510.
244 D. Lavene. Y.A. Weiss, M.E. Safar, Y. Loria, N. Agorus, D. Georges and P.L. Milliez, J. Clin. Pharmacol., 17 (1977) 501-508.
245 M.E. Safar, N.P. Chau, J.A. Levenson A.C., Simon and Y.A. Weiss, Clin. Sci. Molec. Med., 55 (1978) 275S-277S.
246 S. Øie and G. Levy, Eur. J. Clin. Pharmacol., 9 (1975) 115-116.
247 A. Roux, P. Aubert, J. Guedon and B. Flouvat, Eur. J. Clin. Pharmacol., 17 (1980) 339-348.
248 F. Andreasen, P. Jakobsen, H.J. Kornerup, E.B. Pedersen and O.L. Pedersen, Clin. Pharmacol. Ther., 33 (1983) 10-18.
249 M. Safar, Y. Weiss. N.P. Chau, A. Kheder, J. Levenson and P. Milliez, Nouv. Presse méd., 7 (1978) 2759-2760.
250 D.T. Lowenthal. W.A. Briggs, T.P. Gibson, H. Nelson and W.J. Cirksena, Clin. Pharmacol. Ther., 16 (1974) 761-769.
251 F.D. Thompson, A.M. Joekes and D.M. Foulkes, Brit. Med., J. 2 (1972) 434-436.
252 W. Kirch, H. Köhler, G. Berggren and W. Braun, Clin. Nephrol., 18 (1982) 88-94.
253 W.J. Stone and T. Walle, Clin. Pharmacol. Ther., 28 (1980) 449-455
254 R.G. Wilkinson and R.A. Branch, in L.Z. Benet, N. Massoud and J.G. Gambertoglio (Editors), Pharmacokinetic Basis for Drug Treatment, Raven Press, New York, 1983, pp. 49-61.
255 W. Kirch, M. Schäfer-Korting, E. Mutschler, E.E. Ohnhaus and W. Braun, J. Clin. Pharmacol., 23 (1983) 171-177.
256 E.A. Sotaniemi. M. Anttila, A. Rautio, J. Stengard, P. Saukko and P. Järvensivu, Clin. Pharmacol Ther., 29 (1981) 705-710.
257 I. Rocher, S. Decourt, A. Leneveu, D. Lebree, S.P. Rosier and B. Flouvat, Int. J. Clin. Pharmacol. Ther. Toxicol., 23 (1985) 406-410.
258 R. Zaman, D.B. Jack, M.R. Wilkins and M.J. Kendall, Biopharm. Drug Dispos., 6 (1985) 131-137.
259 C. Seyfried, H. Ledermann, H. Rennekamp. M. L'Age and U. Abshagen, Deut. Med. Wschr., 107 (1982) 21-26.
260 C.Y. Quon and H.F. Stampfli, Drug Metab. Dispos., 13 (1985) 420-424.
261 K.N. Buchi, D.E. Rollins, K.G. Tolman, R. Achari, D. Drissel and J.D. Hulse, J. Clin. Pharmacol., 27 (1987) 880-884.
262 J.F. Thiercelin, G. Bianchetti, P. Padovani, P.L. Morselli, D. Fries, J.M. Costa, J. Paccalin, J.Z. Bouchet and C. Martin-Dupont, in L.Z. Benet, G. Levy and B.L. Ferraiolol (Editors), Pharmacokinetics: a modern view, Plenum Press, New York, London 1984, pp. 481-483.

263 W. Kirch, I. Rose, H.G. Demers, G. Leopold, J. Pabst and E.E.
    Ohnhaus, Clin. Pharmacokin., 13 (1987) 110-117.
264 E.E. Ohnhaus, U. Münch and J. Meier, Schweiz. med. Wschr., 106
    (1976) 1748-1750.
265 C.G. Regårdh, L. Jordö, M. Ervik, P. Lundborg, R. Olsson and
    O. Rönn, Clin. Pharmacokin., 6 (1981) 375-388.
266 A.J.J. Wood, D.M. Kornhauser, G.R. Wilkinson, D.G. Shand and
    R.A. Branch, Clin. Pharmacokin., 3 (1978) 478-487.
267 M.J.P. Arthur, A.R. Tanner, C. Patel, R. Wright, A.G. Renwick
    and C.F. George, Gut, 26 (1985) 14-19.
268 R.A. Branch, J. James and A.E. Read, Br. J. Clin. Pharmacol.,
    3 (1976) 243-249.
269 J. Feely, Clin. Pharmacokin., 8 (1983) 1-16.
270 A. Aro, M. Anttila, T. Korhonen and H. Sundquist, Eur. J.
    Clin. Pharmacol., 21 (1982) 373-377.
271 R. Wilkinson and W.A. Burr, Am. Heart J., 108 (1984) 1160-
    1167.
272 B. Hallengren, O.R. Nilsson, B.E. Karlberg, A. Melander, L.
    Tegler, E. Wåhlin-Boll, Eur. J. Clin. Pharmacol., 21 (1982)
    379-384.
273 P.G. Wells, J. Feely, G.R. Wilkinson and A.J.J. Wood, Clin.
    Pharmacol. Ther., 33 (1983) 603-608.
274 T. Ishizaki, M. Masuno and K. Tawara, Res. Comm. Chem. Pathol.
    Pharmacol., 29 (1980) 473-485.
275 G.M. London, M.E. Safar, Y.A. Weiss and P.I. Milliez, J. Clin.
    Pharmacol., 216 (1976) 174-183.
276 K.M. Piafsky, Clin. Pharmacokin., 5 (1980) 246-262.
277 K.M. Piafsky and O. Borga, Clin. Pharmacol. Ther., 22 (1977)
    545-549.
278 O. Borga, K.M. Piafsky and G. Nilsen, Clin. Pharmacol. Ther.,
    22 (1977) 5390-544.
279 G. Sager, O.G. Nilsen and S. Jacobsen, Biochem. Pharmacol., 28
    (1979) 905-911.
280 B.J. Scott, A.R. Bradwell, R.E. Schneider and H. Bishop,
    Lancet, 1 (1979) 930.
281 S. Glasson, R. Zini, P. D'Athis, J.P. Tillement and J.R.
    Boissier, Mol. Pharmacol., 17 (1980) 187-191.
282 F.M. Belpaire, M.G. Bogaert and M. Rosseneu, Eur. J. Clin.
    Pharmacol., 22 (1982) 253-256.
283 L. Šoltés, F. Brée, B. Sebille, J.P. Tillement, M. Ďurišová
    and T. Trnovec, Biochem. Pharmacol., 34 (1985) 4331-4334.
284 K.M. Piafsky, O. Borga, I. Odar-Cederelof, C. Johansson and F.
    Sjöqvist, New Engl. J. Med., 299 (1978) 1435-1439.
285 D.G. McDevitt, M. Frisk-Holmberg, J.W. Hollifield and D.G.
    Shand, Clin. Pharmacol. Ther., 20 (1976) 152-157.
286 J.W. Paxton and R.H. Briant, Br. J. Clin. Pharmacol., 18
    (1984) 806-810.
287 F.P. Abramson, J. Jenkins and Y. Ostchega, Clin. Pharmacol.,
    Ther. 32 (1982) 659-663.
288 R.E. Schneider and H. Bishop, Clin. Pharmacokin., 7 (1982)
    281-284.
289 W. Kirch, H. Spahn, E.E. Ohnhaus and H. Koehler, Biopharm.
    Drug Dispos., 4 (1983) 73-81.
290 R.E. Schneider, H. Bishop, M.J. Kendall and C.P. Quarterman,
    Int. J. Clin. Pharmacol. Ther. Toxicol., 19 (1981) 158-162.
291 J.P. Tillement, F. Lhoste and J.F. Giudicelli, Clin. Pharmaco-
    kin., 3 (1978) 144-154.
292 S.L. Bowman, S.A. Hudson, G. Simpson, J.F. Munro and J.A.
    Clements, Br. J. Clin. Pharmacol., 21 (1986) 529-532.

293 G. Cheymol, J.M. Poirier, J. Barre, A. Pradalier and J. Dry,
    J. Clin. Pharmacol., 27 (1987) 874-879.
294 V. Jagadeesan and K. Krishnaswamy, Eur. J. Clin. Pharmacol.,
    27 (1985) 657-659.
295 M.J. Kendall, D.B. Jack, S.J. Laugher, J. Lobo and S.R. Smith,
    Br. J. Clin. Pharmacol., 18 (1984) 331-335.
296 B. Rosenkranz, H. Ledermann and J.C. Fröhlich, J. Cardiovasc.
    Pharmacol., 8 (1986) 943-949.
297 D.B. Jack, M.J. Kendall, S. Dean, S.J. Laugher and R. Zaman,
    Biopharm. Drug Dispos., 3 (1982) 47-54.
298 A.J. McLean, H. Skews, A. Bobik and F.J. Dudley, Clin.
    Pharmacol. Ther., 27 (1980) 726-732.
299 A.M. Dart, C.S. Chiang, M.E. Ellis, G.M. Hawksworth, T.A.
    Jeffers, K. Parry, A.K. Scott and J.C. Petrie, Br. J. Clin.
    Pharmacol., 13 (1982) 587-588.
300 F. Wagner, D. Kalusche, D. Trenk, E. Jähnchen and H. Roskamm,
    Br. J. Clin. Pharmacol., 24 (1987) 213-220.
301 P. Fenster, D. Perrier, M. Mayersohn and F.I. Marcus, Clin.
    Pharmacol. Ther., 27 (1980) 450-453.
302 J.L. Holtzman, D.C. Kvam, D.A. Berry, L. Mottonen, G. Borrell,
    L.I. Harrison and G.J. Conard, Eur. J. Clin. Pharmacol., 33
    (1987) 97-99.
303 M.G. Bogaert, M.T. Rosseel and R.A. Lefebvre, Br. J. Clin.
    Pharmacol., 17 (1984) 90S-91S.
304 C.G. Regårdh, P. Lundborg and B.A. Persson, Biopharm. Drug
    Dispos., 2 (1981) 79-87.
305 W. Kirch, I. Rose, I. Klingmann, J. Pabst and E.E. Ohnhaus,
    Eur. J. Clin. Pharmacol., 31 (1986) 59-62.
306 E. Rey, P. Jammet, P. D'Athis, D. DeLauture, B. Christoforov,
    S. Weber and G. Olive, Arzneim.-Forsch., 37 (1987) 953-956.
307 H. Spahn, W. Kirch and E. Mutschler, Br. J. Clin. Pharmacol.,
    15 (1983) 500.
308 J.J.R. Houtzagers, O. Streuman and C.G. Regårdh, Br. J. Clin.
    Pharmacol., 14 (1982) 67-72.
309 W. Kirch, H. Kohler, H. Spahn and E. Mutschler, Lancet, ii
    (1981) 531-532.
310 W. Kirch, H. Spahn, H. Kohler, E.E. Ohnhaus and E. Mutschler,
    Klin. Wschr., 60 (1982) 1401-1407.
311 H. Spahn, W. Kirch, P. Hajdu, E. Mutschler and E.E. Ohnhaus,
    Eur. J. Clin. Pharmacol., 29 (1986) 555-560.
312 W. Kirch, H. Spahn, H. Koehler and E. Mutschler, Arch.
    Toxicol. (suppl. 6) (1983) 379-383.
313 T.K. Daneshmend and C.J.C. Roberts, Lancet, i (1981) 565.
314 I.W. Reimann and J.C. Fröhlich, Naunyn-Schmiedeberg's Arch.
    Pharmacol., 316 (suppl.) (1981) 1279, Abstr. 315.
315 I.W. Reimann, U. Klotz, B. Siems and J.C. Fröhlich, Br. J.
    Clin. Pharmacol., 12 (1981) 785-790.
316 A.M. Heagerty, M.A. Donovan, C.M. Castleden, J.F. Pohl, L.
    Patel and A. Hedges, Br. Med. J., 282 (1981) 1917-1919.
317 M.A. Donovan, A.M. Heagerty, L. Patel, C.M. Castleden and J.F.
    Pohl, Lancet, 1 (1981) 164.
318 J. Feely, G.R. Wilkinson and A.R. Wood, Circulation, 62 (1980)
    982-985.
319 A. Markiewicz, M. Hartleb, A. Lelek, H. Boldys and A. Nowak,
    Pol. Arch. Med. Wewn., 73 (1985) 154-159.
320 J. Feely, G.R. Wilkinson and A.R. Wood, New Engl. J. Med, 304
    (1981) 692-695.
321 A.M. Heagerty, C.M. Castleden and L. Patel, Br. Med. J., 284
    (1982) 1304.

322 L.Patel and K. Weerasuriya, Br. J. Clin. Pharmacol., 15 (1983) 152-154.
323 W. Reimann, U. Klotz and J.C. Fröhlich, Clin. Pharmacol., Ther. 32 (1982) 749-757.
324 H. Spahn, E. Mutschler, W. Kirch, E.E. Ohnhaus and H.D. Janisch, Br. Med. J., 286 (1983) 1546-1547.
325 J.G. Kelly, R.G. Shanks and D.G. McDevitt, Br. Med. J., 287 (1983) 1218.
326 W. Kirch, M. Schäfer-Korting, T. Axthelm, H. Köhler and E. Mutschler, Clin. Pharmacol. Ther., 30 (1981) 429-435.
327 J.H. Dobbs, V.A. Skoutakis, S.R. Acchiardo and B.R. Dobbs, Curr. Therap. Res., 21 (1977) 887-892.
328 S.A. Babhair and M. Tariq, Pharmacol. Res. Commun., 16 (1984) 845-850.
329 J. McAinsh, B.H. Holmes, T.J. Fitzsimons and J. Young, Biopharm. Drug Dispos., 7 (1986) 223-231.
330 J. McAinsh, W. Bastain, J. Young and J.D. Harry, Biopharm. Drug Dispos., 2 (1981) 147-156.
331 E. Riva, P.L. Farina, R. Sega, G. Tognoni, W. Bastain and J. McAinsh, Eur. J. Clin. Pharmacol., 17 (1980) 333-337.
332 W. Krause and C. Lennert, Biopharm. Drug Dispos., 4 (1983) 339-345.
333 L. Jordö, G. Johnsson, P. Lundborg, B.A. Persson, C.G. Regårdh and O. Rönn, Br. J. Clin. Pharmacol., 7 (1979) 563-567.
334 K. Felder, H.E. Geibler, S. Hiemstra, E. Mutschler, M. Schäfer and E. Ziegler, Arzneim.-Forsch., 29 (1980) 1746-1752.
335 A. Roux, A. LeLiboux, B. Delhotal, J. Gaillot and B. Flouvat, Eur. J. Clin. Pharmacol., 24 (1983) 801-806.
336 J. McAinsh, B.F. Holmes, N.S. Baber and J. Young, Biopharm. Drug Dispos., 2 (1981) 167-175.
337 M. Chiariello, M. Volpe, F. Rengo, B. Trimarco, R. Violini, B. Ricciardelli and M. Condorelli, Clin. Pharmacol. Ther., 26 (1979) 433-436.
338 W. Kirch, H. Spahn, N.R. Kitteringham, H.J. Hutt, E. Mutschler and E.E. Ohnhaus, Br. J. Clin. Pharmacol., 17 (1984) 65S-68S.
339 G. Alvan, K. Piafsky, M. Lind and C. von Bahr, Clin. Pharmacol. Ther., 22 (1977) 316-321.
340 K. Haglund, P. Seideman, P. Collste, K.O. Borg and C. von Bahr, Clin. Pharmacol. Ther., 26 (1979) 326-329.
341 R.E. Vestal, D.M. Kornhauser, J.W. Hollifield and D.G. Shand, Clin. Pharmacol. Ther., 25 (1979) 19-24.
342 R.J. Herman, K. Nakamura, G.R. Wilkinson and J.J. Wood, Pharmacologist, 24 (1982) 181.
343 P.N. Bennett, V.A. John and V.B. Whitmarsh, Br. J. Clin. Pharmacol., 13 (1982) 387-391.
344 M. Schäfer-Korting, W. Kirch, T. Axthelm, H. Köhler and E. Mutschler, Clin. Pharmacol. Ther., 33 (1983) 283-288.
345 H. Spahn, P. Langguth, D. Krauss, W. Kirch and E. Mutschler, Arch. Pharmacol. (Weinheim), 320 (1987) 103-107.
346 P. DeSouich, G. Caille and P. Larochelle, Clin. Pharmacol. Ther., 33 (1983) 585-590.
347 S. Kärkkäinen and P.J. Neuvonen, Int. J. Clin. Pharmacol. Ther. Toxicol., 22 (1984) 441-446.
348 M.J. Kendall, C.P. Quarterman, D.B. Jack and L. Beeley, Br. J. Clin. Pharmacol., 14 (1982) 120-122.
349 M.J. Kendall, D.B. Jack, C.P. Quarterman, S.R. Smith and R. Zaman, Br. J. Clin. Pharmacol., 17 (1984) 87S-89S.
350 S.K. Gardner, W.J. Cady and Y.S.Ong, Int. J. Clin. Pharmacol. Ther. Toxicol., 18 (1980) 421-424.

351 T. Walle, U.K. Walle, T.D. Cowart, E.C. Conradi and T.E. Gaffney, J. Pharmacol. Exp. Ther., 241 (1987) 928-933.
352 L.J. Schaaf, S.C. Campbell, M.B. Mayersohn, T. Vagedes and D.G. Perrier, Eur. J. Clin. Pharmacol., 33 (1987) 355-361.
353 G. Johnsson, L. Jordö, P. Lundborg, C.G. Regårdh and O. Rönn, Int. J. Clin. Pharmacol. Ther. Toxicol., 18 (1980) 292-297.
354 J.H. Silas, S. Freestone, M.S. Lennard and L.E. Ramsay, Br. J. Clin. Pharmacol., 20 (1985) 387-391.
355 M.J. Kendall, D.B. Jack, K.L. Woods, S.J. Laugher, C.P. Quarterman and V.A. John, Br. J. Clin. Pharmacol., 13 (1982) 393-398.
356 G. Ragnarsson, A. Sandberg, U.E. Jonsson and J. Sjögren, Drug Devel. Indust. Pharm., 13 (1987) 1495-1509.
357 J. McAinsh, N.S. Baber, R. Smith and J. Young, Br. J. Clin. Pharmacol., 6 (1978) 115-121.
358 W.J. Leahey, J.D. Neil, M.P. Varma, R.G. Shanks, E. Perucca, R. Grimaldi, G. Gatti, M. Caravaggi and F. Crema, Br. J. Clin. Pharmacol., 9 (1980) 33-40.
359 O.H. Drummer, J. McNeil, E. Pritchard and W.J. Louis, J. Pharm. Sci., 70 (1981) 1030-1032.
360 J. McAinsh, N.S. Baber, B.F. Holmes, J. Young and S.H. Ellis, Biopharm. Drug Dispos., 2 (1981) 39-48.
361 E. Perucca, G. Grimaldi, G. Gatti, M. Caravaggi, F. Crema, S. Lecchini and G.M. Frigo, Br. J. Clin. Pharmacol., 18 (1984) 37-43.
362 M.J. Serlin, M.L.E. Orme, M. Mac Iver, G.J. Green, R.G. Sibeon and A.M. Breckenridge, Br. J. Clin. Pharmacol., 15 (1983) 519-527.
363 D. Dvornik, M. Kraml, J. Dubuc, S. Patterson-Kreuscher, G. Milosovich and J.F. Mullane, J. Clin. Pharmacol., 21 (1981) 472-476.
364 G.S. Nace and A.J.J. Wood, Clin. Pharmacokin., 13 (1987) 51-64.
365 A. Bobik, G.L. Jennings, P.I. Korner, P. Ashley and G. Jackman, Br. J. Clin. Pharmacol., 7 (1979) 545-549.
366 R. Johansson, C.G. Regårdh and J. Sjögren, Acta Pharmac. Suecica, 8 (1971) 59-70.
367 D.B. Jack, M.J. Kendall, S.J. Laugher and S.R. Smith, Br. J. Clin. Pharmacol., 19 (1985) 185S-190S.
368 K.L. Woods, D.B. Jack, M.J. Kendall and A. Halsey, M.L. O Donnel, S.J. Warrington and V.A. John, Br. J. Clin. Pharmacol., 19 (1985) 177S-184S.
369 J. Marinow, W. Akpan, H. Ledermann and U. Abshagen, Z. Kardiol. Angiol. Klin. Praxis, 11 (1981) 548-554.

Chapter 3

SAMPLE PRETREATMENT IN THE DETERMINATION OF BETA-BLOCKERS IN BIOLOGICAL FLUIDS

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3.1 INTRODUCTION

Two main categories of analysis can be distinguished based on the yield of medical information (ref. 1):

- analysis performed on substances that are thought to be associated with a consequence rather than the cause of disease, i.e. determination of urea, creatinine, total proteins, cholesterol, sodium, potassium, calcium, glucose, etc.;
- analysis performed on substances that are thought to be associated with the cause rather than the consequence of illness, i.e., determination of special proteins, hormones, vitamins, antibodies and drugs which can reflect specific microbial invasion, drug toxicity or malfunction of the immune system, etc.

Substances in the first group occur at relatively high concentrations (10^{-4} mol 1^{-1}) and can be measured by relatively simple methods. On the other hand, the determination of substances in the second group is more complex as smaller amounts must be specifically determined in the presence of a complicated biological matrix.

Consequently, the determination of drugs, which belong to the second group, is a complex procedure that can be readily divided into three main steps (Fig. 3.1):

- converting the biological sample into the analytical sample, i.e., pre-measurement step,
- obtaining the analytical signal, i.e., measurement step,
- evaluating the analytical signal, i.e., post-measurement step.

Until recently, attention was paid mainly to the second and third steps. During the past two decades, increasingly sensitive

BIOLOGICAL SAMPLE

extraction
chem. modification

ANALYTICAL SAMPLE

measurement

ANALYTICAL SIGNAL

math. evaluation

RESULT
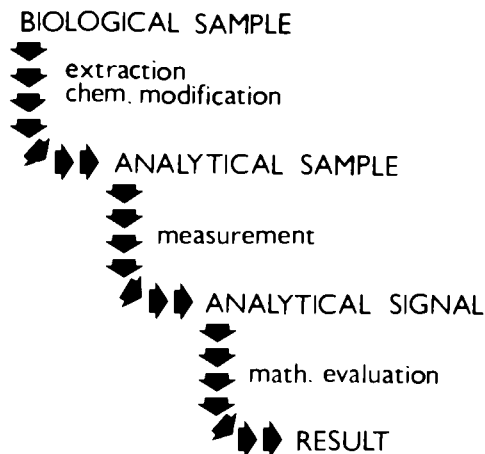
Fig. 3.1. Three steps in the determination of drugs

and sophisticated methods, techniques and devices have been inven-
ted. The development of sensitive, specific and stable detectors
in HPLC and GLC and the introduction of immunoanalytical methods
have rendered possible the determination of femtomole concentra-
tions of drugs. Advances in column technology in HPLC and GLC have
allowed these concentrations to be measured in more complex mixtu-
res. The widespread utilization of microprocessor techniques in
chromatographic and optical methods not only reduced the manual
calculations, and thus simplified the evaluation of the analytical
signal, but also allowed the whole process from sampling to evalu-
ation to be unified into one operation. Thus, the three-step
procedure for the determination of drugs outlined in Fig. 3.1,
could be simplified into a two-step procedure, i.e. the prepara-
tion of the sample and its instrumental analysis. The trend in
analytical practice has evidently been moving both towards increa-
sing productivity and freeing staff from mundane tasks and towards
the detection and quantification of analytes at even lower concen-
trations.

In comparison with this development, almost no attention has
been paid to the preparation of the analytical sample. In most
determinations, there are still many laborious stages involved in
the conversion of the biological sample into the analytical

sample. Moreover, the stages have remained the same as they were at the initial period of the bioanalysis of drugs. Beta-blockers, and particularly propranolol, can serve as good examples of this "fossilized" state. As mentioned in the Introduction to this book, the development of methods for the determination of propranolol in biological material started in the mid 1960s with optical methods; at beginning of the 1970s attention shifted from spectrofluorometry to gas-liquid chromatography and by the end of 1970s from GLC to HPLC. Despite this development, and despite developments within the individual techniques, the procedure for the isolation of propranolol from blood samples has remained almost unchanged. In the work of Back and co-workers in 1965 (ref. 2), blood samples were alkalinized with a solution of sodium hydroxide and extracted with an organic solvent, heptane containing 1% of ethanol. After a back-extraction, the acidic solution then served for the determination of propranolol spectrofluorometrically. Ten years ago, in a GLC method for propranolol described by Walle and co-workers (ref. 3), only some material was changed but not the procedure. Plasma was used as a body fluid in the experiment, alkalinization was effected with carbonate buffer and extraction was performed with ethyl acetate. In their HPLC determination of propranolol published in 1986, Yamamura and co-workers alkalinized plasma with a solution of NaOH and extracted the mixture with heptane-isoamyl alcohol (98.5:1.5) (ref. 4). Hence, more than twenty years passed without substantial changes.

The only actual advancement in sample preparation has been achieved by applying knowledge from the chemistry of bonded silica phases to the extraction of substances from a biological matrix. Although this type of extraction, called liquid-solid or solid--phase extraction, has been gaining wider acceptance only since the beginning of the 1980s, its advantages, i.e., simplicity, efficiency, reproducibility, suitability for automation, etc., have already turned it into a serious challenge to conventional solvent extraction.

In the pretreatment, without respect to the mode of performance, three main variables must be taken into account:
- the drug and its physico-chemical properties,
- the biological matrix and its complexity, and
- the instrumental method with its sensitivity and selectivity.

3.2 STRUCTURE OF BETA-BLOCKERS

There are two main physico-chemical properties of drug to be
considered before a method for their determination in biological
material is developed: acid-base properties (pK_a) and hydrophobi-
city (log P).

The beta-blocker molecule consists of four main parts (Fig.
3.2) which influence its physico-chemical properties (and also its
pharmacological properties):
- aromatic part,
- oxypropanolamine or ethanolamine bridge,
- secondary amino group,
- terminating alkyl group, but in some beta-blockers, e.g., labe-
  talol or tolamol, continuing to another structure.

$$Ar-O-\underset{\underset{OH}{|}}{CH_2}-CH-CH_2-NH-R \qquad\qquad Ar-\underset{\underset{OH}{|}}{CH}-CH_2-NH-R$$
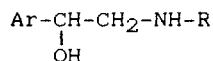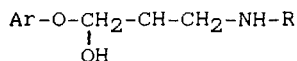
Fig. 3.2. Structures of beta-blockers (see also Supplement).

In most instances, the secondary amino group is the most
easily dissociable group in the structure of the drugs with pK_a
values expected in the range 8.0 - 8.5 (ref. 5). The higher pK_a
values actually measured for beta-blockers, i.e., of the order of
about 1 unit of pK_a (Table 3.1), are caused by hydrogen bonds
formed between the amino group and the hydroxy group in a vicinal
position (ref. 5).

There are a wide variety of substituents on the aromatic ring
of beta-blockers (more than ten chemical groups) and their amounts
(from mono- to tetra-substitution), and there are also different
types of aromatic systems involved in this group (phenyl,
naphthyl, condensed and non-condensed heterocyclics, etc.). How-
ever, owing to the alkyl bridge with its insulating property, the
aromatic ring system has only a small effect on the amino group of
the alkanol side-chain of beta-blockers (ref. 6). Therefore, the
acid dissociation constants reported for the drugs of this group
do not differ substantially from the mean value of 9.4 (Table
3.1). With sotalol, the second dissociation constant (dissociation
of the proton of SO_2-NH group) has also been reported (pK_a=8.3)
(ref. 5).

Although the aromatic part of the molecules plays a minor

role in influencing dissociation of the side-chain amino group, it is the major structural part of beta-blockers in relation to their lipophilicity. Owing to the wide variety of aromatic systems involved in the structure of beta-blockers (see Supplement), their lipophilicities cover six orders of octanol/water log P values (Table 3.1).

TABLE 3.1

pK_a values and lipophilicity characteristics for 15 beta-blockers (refs. 5, 7-15).

Drug	pK_a	log D[a]	log P
Acebutolol	9.2	0.09	1.87
Alprenolol	9.6	1.16	2.61
Atenolol	9.32	-1.64	0.23
Bunolol	9.32	0.47[b]	2.40
Carteolol	9.75	-0.68	1.67[b]
Labetalol	9.45	1.06	3.18
Metoprolol	9.24	0.13	2.15
Nadolol	9.39	-1.18	0.71
Oxprenolol	9.32	0.67	2.18
Penbutolol	9.26	1.59[c]	4.15
Pindolol	9.46	-0.09	1.75
Practolol	9.44	-0.97	0.79
Propranolol	9.23	1.51	3.65
Sotalol	9.65	-1.82	-0.79
Timolol	9.21	0.06	2.10

[a] Logarithm of the distribution coefficient between octanol and phosphate buffer (pH 7.4).

[b] Calculated from the known pK_a and either the distribution or partition coefficient.

[c] At pH 7.

As monobasic acids, these drugs exist at physiological pH as singly charged cations and only approximately 1% of the total drug concentration at this pH is present in the undissociated form. This can also influence the extraction of beta-blockers by organic solvents, which is possible only in undissociated form. Therefore,

alkalinization to $pH > pK_a$ is necessary for transforming cations into undissociated bases and thus improving their extractability (preferably $pH-pK_a > 2$).

3.3 BIOLOGICAL FLUIDS

As already mentioned, beta-blockers have been determined in biological material for almost 25 years, and the proportions reported in various kinds of biological fluids are summarized in Table 3.2.

TABLE 3.2

Representation of different kinds of biological fluids in the analytical literature devoted to the determination of beta--blockers.

Biological fluids	%
Plasma	47
Urine	25
Serum	7
Whole blood	7
Breast milk	3
Saliva	1
Other body fluids[a]	3

[a]Amniotic fluid, bile, cerebrospinal fluid, dialysis fluid, ocular liquid, seminal plasma, tears.

In drug analysis, the most commonly used biological material has been blood analysed mostly as plasma or serum. In the determination of beta-blockers, almost half of all methods published concern plasma concentrations. Serum and whole blood have been used much less frequently. These three liquids represent 60% of the literature devoted to the determination of beta-blockers.

The chief feature of plasma and serum is the presence of large amounts of proteins. Binding of drugs to these proteins leads to a subdivision of the "total" drug present in plasma or serum in "bound" and "free" fractions. The bound/free ratio de-

pends on the amount of proteins - in the case of beta-blockers mainly on the amount of $_1$-acid glycoprotein - and on the properties of drugs - in beta-blockers on their lipophilicity. This ratio can be affected by materials used for handling blood samples. The use of plastic materials containing plasticizers, such as tris(2-butoxyethyl)phosphate or di(ethylhexyl) phthalate, causes displacement of beta-blockers , and some other basic drugs, from $_1$-acid glycoprotein (refs. 16, 17). Storing whole blood containing propranolol in unsuitable material led to spuriously low plasma concentrations of this drug (ref. 18). Although this problem has been avoided by using materials free from tris(2--butoxyethyl)phosphate (ref. 19), work with blood samples in all--glass equipment is recommended. A decrease in concentrations caused by binding to a glass surface can be prevented by silanization (ref. 20).

Determination of beta-blockers in urine is mostly connected with the determination of their metabolites. As basic extracts of urine are not as heavily contaminated with endogenous compounds as are acidic extracts (ref. 21), its processing before the analysis of unchanged beta-blockers does not differ greatly from the treatment of plasma or serum. In some instances, unprocessed urine can be used directly for the determination of the drugs. Hence, its dilution with mobile phase and the subsequent injection of the resulting solution was used for the HPLC determination of atenolol, bisoprolol and metoprolol in human urine (ref. 22).

In the last few years, there has been strong interest in saliva as a potential substitute for plasma and serum, mainly for therapeutic monitoring of drugs. Measurement of the drug concentration in saliva offer several advantages, e.g., no discomfort for patients, no trained personnel required for sampling and measurement of the free fraction of drug (ref. 23). However, with beta-blockers, the prerequisite for this substitution, i.e., a well defined, concentration-independent and constant relationship between the drug concentration in saliva and in plasma or serum, has frequently not been fulfilled. As a result, both inter- and intra-individual variations have been reported (ref. 24).

The determination od beta-blockers in other biological materials has only rarely been studied, mostly when some special effects of the drugs were being investigated. In addition to their effect on the cardiovascular system, some beta-blockers , e.g., timolol, also have therapeutic value in ophtalmology in the treat-

ment of glaucoma (ref. 25). To study the transport of these drugs after topical administration, their concentrations in ocular liquid had to be determined (ref. 26).

The measurement of beta-blockers concentrations in cerebrospinal fluid has been connected with studies of their effect on the central nervous system (ref. 27).

In the determination of propranolol in seminal fluid, in connection with a possible spermicidal activity of propranolol, the concentrations of the drug were approximately the same as in serum, and the seminal plasma/serum ratio was independent of time (ref. 24).

Beta-blockers have also been widely prescribed in the management of hypertension during pregnancy and lactation (ref. 28). To estimate possible effects on foetuses and newborns, determinations of these drugs in amniotic fluid, cord plasma and breast milk have been performed (ref. 29). As the main difference between breast milk and other biological fluids is a relatively high concentration of fatty acids and related lipids in the former, delipidation of this fluid prior to extraction was performed in some instances (ref. 30).

As the enterohepatic circulation is regarded an important factor in the fate of beta-blockers in livig organisms, their concentration in bile has been studied (ref. 31). In this instance, extraction of the drugs can be influenced by the formation of ion pairs between beta-blockers and bile acids (ref. 32).

3.4 PREPARATION OF THE ANALYTICAL SAMPLE

Each of the biological materials discussed in the previous section represents a complex medium in which the drug of interest is present at extremely low concentrations compared with the endogenous components. In most instances, such samples cannot be directly subjected to an analytical procedure. Therefore, conversion of the biological sample into an analytical sample is necessary.

This sample preparation should serve two purposes:
- selective isolation of the drug from the biological material,
- concentration of the drug.

The isolation of the drugs, or the clean-up procedure, is the most important part of the pre-measurement step in the determination of a drug. Also, with the advent of recent "over-micropro-

cessored" devices it remains the only procedure in which the analyst comes into contact with the material to be analysed rather than being just a supervisor of the expensive, fully automatic instrument.

The degree of clean-up itself is dependent on two factors, i.e., the analytical method used and the concentration of the drug in the biological material. From the point of view of the method, two other factors determine the extent of the clean-up procedure: the specificity of the analytical method and the tolerance of the system to contamination.

From the point of view of these conditions, there is almost no method for the determination of beta-blockers whose selectivity, specificity and resistance towards contamination would enable it to be used directly for the determination without a prior clean-up step. Even if the methods are, in principle, satisfactorily selective and sensitive, e.g., capillary GLC with electron-capture detection, they are frequently subject to contamination (proteins for capillary GLC columns and almost everything for ECD), and hence careful isolation, which should prevent contamination, must be performed. On the other hand, methods that are sufficiently resistant towards contamination by endogenous substances, e.g., saturation methods, are not sufficiently selective for metabolites of beta-blockers and the co-determination of these metabolites has frequently occurred.

Despite these problems some papers have described the direct determination of beta-blockers in biological samples. In all of them, saturation methods have been used. However, also in these cases the incorporation of a sample preparation step may improve both the sensitivity and selectivity, as follows.

In the radioimmunoassay of propranolol using the ring-bonded propranolol-bovine serum albumin complex as the antigen (ref. 33), the cross-reactivity with the 4-hydroxy metabolite was higher than 50%. In order to eliminate the contribution of this metabolite, the samples were extracted with toluene.

In the enzyme immunoassay of befunolol (ref. 34), precipitation of plasma proteins with acetone was used and the drug was subsequently succinylated before the assay itself. By using this sample preparation 30 pg of befunolol could be detected and the cross-reactivity with the main metabolite was 0.04%.

A three-step liquid-liquid extraction of cetamolol prior to radioreceptor assay (RRA) (ref. 35) improved the sensitivity of

the assay from 31 to 1 ng ml^{-1} and also resulted in a good agreement between RRA and HPLC determinations of the drug.

3.4.1 Non-extraction methods

By using these methods, increased selectivity and/or lower contamination is mainly achieved by chemical changes of the impurities present in a biological sample. These chemically changed impurities are either removed easily from the system, e.g., denatured proteins, or they are undetectable by the detection system used, e.g., oxidized metabolites. In the determination of beta--blockers, this type of clean-up procedure has not been used very frequently. Its efficiency is not as high as that of extraction methods. With beta-blockers, liquid chromatography and radioreceptor assay have been used after non-extraction methods of isolation.

Lo and Riegelman (ref. 36) used the addition of acetonitrile (0.4 ml to 0.2 ml of plasma) and evaporation of the supernatant to a small volume in the determination of propranolol and its 4--hydroxy metabolite. Tang-Liu et al. (ref. 37) used a plasma--acetonitrile ratio of 1:4 and evaporation of the supernatant to dryness in the determination of bunolol and one of its metabolites. In the determination of esmolol, perchloric acid was used for the precipitation of proteins (ref. 38). The supernatant was analysed directly by HPLC. Albani and co-workers (ref. 39) formed a two-phase system from plasma, sodium chloride, sodium carbonate and acetonitrile, of which the acetonitrile-rich phase was injected directly. Detection limits comparable to those obtained by other isolation methods were described and no deterioration of LC columns was mentioned.

Chemical reaction as a step towards removing co-determined metabolites, mainly 4-hydroxypropranolol, was used in the radio-receptor assay of propranolol (ref. 40). The sample was oxidized with hydrogen peroxide, and RRA without and with this oxidation enabled information to be obtained on the concentrations of both propranolol and its metabolite.

3.4.2 Liquid-liquid extraction

Most biological samples containing beta-blockers have been converted into an analysable form with the help of partition, adsorption and absorption, or by a combination of these physical methods. As shown above, during the entire history of the determi-

nation of beta-blockers in biological fluids, and not only of
these drugs and in biological fluids, their partition between
water and an organic phase, i.e., solvent extraction, or liquid-
-liquid extraction, has been the mostly utilized method. Samples
treated by solvent extraction are usually cleaner than those
obtained by protein precipitation, in that they contain smaller
amounts of iterfering substances. Also, as the sample can be
concentrated in the process, the limit of detection is improved.

Although the difference in partition coefficients within this
group are large - penbutolol differs in log P with respect to
sotalol by five orders of magnitude (Table 3.1) - beta-blockers as
a group can be considered to be lipophilic componds. As such, they
can be extracted by various organic liquids. The extraction proce-
dure itself, as part of the sample preparation, is usually a
function of the analytical method used. The options available for
processing body fluids and also tissue homogenates, are outlined
in Fig. 3.3.

As the lipophilicity of these drugs is bound to their non-
-ionized forms, adjustment of the pH to a level exceeding their
pK_a values by ca. 2 units is the first step of each extraction.
For this alkalinization, addition of either a small amount of
concentrated sodium hydroxide, e.g., 0.1 ml of 5 mol l^{-1} solution,
or a large volume of basic buffers, e.g., 1 ml of carbonate
buffer, has been used. The addition of sodium chloride is fre-
quently coupled with the alkalinization of a sample in order to
free the beta-blockers from other compounds in the biological
sample, e.g., proteins.

In chromatographic techniques, the addition of a fixed amount
of an internal standard is used before extraction to permit
correction for losses during sample treatment and to minimize
errors due to variations in instrument response, injection volume
or derivatization yield. To do this, the internal standard must be
added directly to the biological sample and only after this is the
overall extraction procedure started.

Internal standards used in gas-liquid chromatography and
high-performance liquid chromatography of beta-blockers can be
divided into four groups with respect to their similarity to the
drug being analysed. They are, in order of increasing similarity:
- compounds from other chemical groups,
- other drugs from the group of beta-blockers,
- chemical derivatives or analogues of the drugs being analysed,

- deuterated analogues of the drugs being analysed.
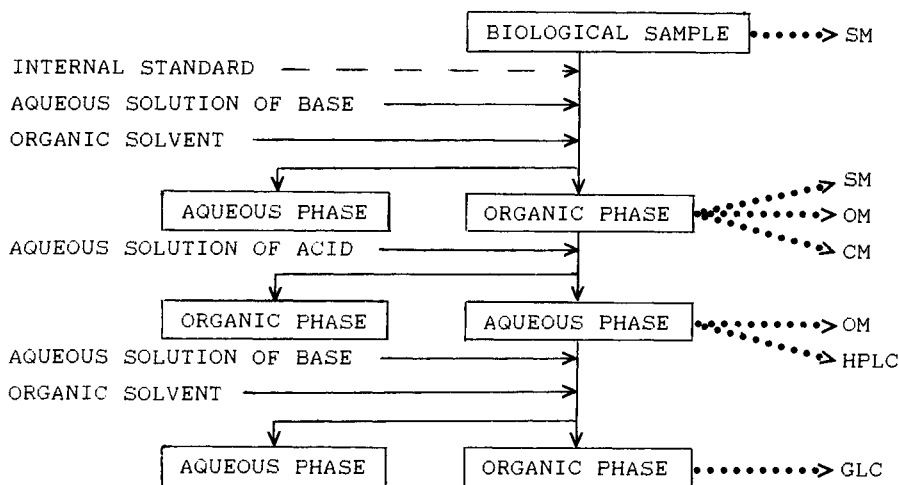
Fig. 3.3. Liquid-liquid extraction procedure for isolation of beta-blockers from biological material. SM = saturation methods; OM = optical methods; CM = chromatographic methods; HPLC = high--performance liquid chromatography; GLC = gas-liquid chromato-graphy.

The internal standards from the first group have been used mainly in the HPLC of beta-blockers. Although compounds of this group do not fulfil one of the requirements for internal standards, i.e., chemical similarity to the substance of interest (ref. 41), their similar chromatographic properties to the being drug analysed may look attractive. However, different extraction properties of beta-blockers and internal standards of this group can cause complications in the determination. Also, owing to differences in the optical properties of penbutolol and quinidine, used as the internal standard in the TLC of the beta-blocker, each TLC plate had to be scanned twice (ref. 42).

Most internal standars used for the determination of beta--blockers by chromatographic methods belong to the second and the third groups. Various beta-blockers and their derivatives and ana-logues have been used as internal standards for other drugs of this group. Two compounds were suggested as suitable internal standards for propranolol - 4-methoxypropranolol (ref. 43) and the

N-cyclopentyldesisopropyl derivative of this drug (ref. 44) - but they have been little used in practice.

Deuterated analogues have been used as internal standards in the selected ion monitoring of several beta-blockers. As they have essentially the same chemical and physical properties as the non--deuterated drugs, they can serve as the ideal compouns for this purpose.

The main task in liquid extractions is, of course, the choice of an organic liquid as a solvent suitable for a particular drug. Owing to the great differences in the lipophilicity of beta--blockers, it has been almost impossible to find one solvent that would be suitable for each of them. The first division from the point of view of extractability of this group of drugs can be found in the paper of Walle (ref. 45) on the gas chromatographic determination of some beta-blockers. Two groups were delineated in his work, i.e., benzene-extracted beta-blockers (propranolol, alprenolol, oxprenolol and dichloroisoproterenol) and ethyl aceta-te-extracted beta-blockers (practolol and sotalol). Winkler and Lemmer, in an effort to develop one standard method for the HPLC determination of beta-blockers (ref. 46), used compound--specific butanol-heptane mixtures in the ratios 1:4, 1:9 and 1:1 for propranolol, metoprolol and atenolol, respectively.

Lefebvre and co-workers (ref. 47) attempted to select the best organic solvent for eight beta-blockers and one metabolite. Their effort is a good example of the fact that searching for the best organic solvent for the whole group, from the point of view of their recovery, means searching for a solvent for the last lipophilic member of the group. With increasing polarity of the solvents used, more and more hydrophilic beta-blockers reached acceptable recoveries, i.e., propranolol already in alkanes, meto-prolol, oxprenolol and timolol in benzene or toluene, pindolol in diethyl ether, acebutolol in chloroform or dichlormethane and atenolol in dichlormetane-1-butanol (4:1) and chloroform-1-penta-nol (3:1). Extraction of sotalol remained incomplete even using these polar mixtures. The last mixture was then concluded to be the best for the whole group. However, with increasing polarity of the solvents, the range of endogenous compounds co-extracted also increases. Therefore, the best organic solvent for the whole group is not necessarily the best for a particular member of the group.

Searching for a group-extraction solvent is substantiated when looking for a suitable extractant for a drug and its metabo-

lites. As metabolites are usually more hydrophilic that the parent drug, it may also apply that the solvent with the best recovery for the most hydrophilic metabolite is the solvent of choice for the whole group (e.g., benzene as the extractant for metoprolol alone, but dichlormethane for the drug and two of its metabolites (ref. 48)).

The influence of the lipophilicity of beta-blockers on the type of extractant is observable, after a closer examination, from the choice of solvent for extraction in particular methods for the determination of the drugs. From the literature the most pre-ferred organic liquids or mixtures used for extraction before the HPLC determination of 16 beta-blockers were selected and are shown in Table 3.3.

In 1980, Hata and co-workers suggested n-butyl chloride as a suitable extractant for various basic drugs, including propranolol (ref. 49). The advantages described were a clear extract and easy separation of phases because of the hydophobic character of the solvent. This suggestion has not found wide acceptance; n-butyl chloride has been used only once for the HPLC determination of metoprolol (ref. 50) and, in a mixture with 10% 1-butanol, for the HPLC of atenolol (ref. 51).

For the second most hydrophilic beta-blocker, atenolol, the addition of heptafluorobutanol to dichloromethane (ref. 52) or methyl tert.-butyl ether (ref. 53) was used to improve the recove-ry.

Although beta-blockers can form ion pairs (ref. 54), this principle has practically not been used for their extraction. The extraction of propranolol from breast milk, after addition of 0.5 ml of 5 mol l^{-1} HCl to 0.5-1.0 ml of breast milk using chloroform--methanol (4:1), is one exception (ref. 55).

Frequently, a single extraction step is not sufficient, so back-extraction has to be performed owing to insufficient clean-liness of such a single extract or to its insuitability for injec-tion into a chromatographic system, or both. In such instances, 1 mol l^{-1} hydrochloric acid has been used in most back-extraction procedures. This solution was used with success for the back--extraction of lipophilic drugs from non-polar solvents, e.g., propranolol from n-hexane (ref. 56), and of less lipophilic drugs from more polar solvents, e.g., atenolol from 30% cyclohexane in butanol (ref. 57). If the first extraction solvent is too polar, a less polar solvent can be added before the back-extraction, e.g.,

TABLE 3.3

Solvents recommended for the extraction of 16 beta-blockers from
biological fluids prior to their HPLC analysis and logarithms of
their partition coefficients.

Drug	Solvent	log P[a]
Penbutolol	n-hexane/n-pentanol, 96:4	4.15
Propranolol	n-alkane[c]/iso pentanol, 98:2	3.65
Labetalol	ethyl acetate	3.18
Alprenolol	diethyl ether	2.61
Betaxolol	diethyl ether	2.59[b]
Bunolol	benzene	2.40
Oxprenolol	dichlorethane	2.18
Metoprolol	dichlormethane	2.15
Timolol	diethyl ether	2.10
Acebutolol	ethyl acetate	1.87
Pindolol	diethyl ether	1.75
Carteolol	chloroform	1.60[b]
Practolol	ethyl acetate	0.79
Nadolol	n-pentane/isopentanol, 4:1	0.71
Atenolol	dichlormethane/n-butanol, 9:1	0.23
Sotalol	chloroform/alkanol[d], 3:1	-0.79

[a] (refs. 7, 10, 13).
[b] Calculated from the distribution coefficient and pK_a
[c] n-alkane = n-pentane, n-hexane, n-heptane.
[d] Alkyl = 1-propyl, 1-butyl, 1-pentyl.

the addition of light petroleum to 1-butyl acetate in the back-
-extraction of nadolol (ref. 58). In addition to hydrochloric
acid, H_2SO_4 and H_3PO_4 have also been used.

   In extraction procedures before gas-liquid chromatographic
determinations a third extraction step is performed after back-
-extraction to transfer the solute back into the organic phase.
After alkalinization, mostly with sodium hydroxide, extraction
with the same solvent as in the firs extraction has mostly been
used. In the GLC determination of nadolol, lyophilization of the
aqueous phase and redissolution of the rest in methanol was
applied instead of the third extraction (ref. 58). Another means

of avoiding a third extraction includes an acid extraction before
the first, basic extraction. This means that the sample is first
acidified to remove part of the co-extractable impurities by
liquid extraction and then alkalinized to extract the drug. This
"opposite" method has been used only occasionally with beta-
-blockers (refs. 59, 60).

3.4.3 Liquid-solid extraction

As mentioned at the beginning of this chapter, liquid-liquid
extraction has persisted for more than 20 years as the main clean-
-up procedure in the determination of beta-blockers. There have
been four main reasons for this long life in comparison with the
striking developments in the instrumental aspects of bioanalysis.

First, a wide variety of different organic solvents provides
the possibility of choosing the best one, from the point of view
of either recovery or selectivity, for each particular drug.
Second, the extract concentration step can be used to increase the
assay sensitivity. Third, laboratory equipment has been designed
to make this procedure as easy as possible (mixers, shakers,
rollers, portable centrifuges, etc.). Finally, there was virtually
no competing method to overcome the disadvantages of liquid
extraction, such as time consumption, large volumes of organic
liquids frequently used, emulsion formation with some organic
liquids, environmental problems in the routine handling of toxic
and flammable solvents, etc.

The classical adsorbents such as charcoal or alumina have
hardly been used for the isolation of beta-blockers from biologi-
cal material. The use of charcoal in the thin-layer chromatogra-
phic determination of sotalol in connection with compliance moni-
toring is an exception. This hydrophilic beta-blocker was extrac-
ted from human urine by mixing the biological fluid with carbona-
te-hydrogencarbonate buffer (pH 10) and a charcoal slurry. Sotalol
was then replaced from the adsorbent with methanol (ref. 61).
Application of buffered celite in columns as an adsorbent in the
forensic toxicology of beta-blockers has also been discussed (ref.
62).

The first widely used solid materials for the extraction of
beta-blockers were styrene-divinylbenzene copolymers, forming non-
-ionic resins with a macroreticular structure and a high area-to-
-volume ratio. Amberlite XAD-2 has been the most utilized type. Of
various kinds of interactions that can occur between drugs and

Amberlite XAD-2, i.e, Van der Waals forces, dipole-dipole inter-
actions and hydrophobic interactions (ref. 63), the last type play
a great role with beta-blockers, as the ability of different drugs
of this group to be extracted by the non-ionic resins correlates
well with their hydrophobic properties (ref. 64).

Recent advances in liquid-solid extraction methods in sample
preparation have been connected with the development of new mate-
rials for HPLC packings, particularly of covalently bonded statio-
nary phases. This method of sample preparation has been reported
in an increasing number of papers dealing with bioanalysis, and in
catalogues from different chromatographic manufacturers, indi-
cating an actual trend rather than a fad. Variously modified
silica phases were adopted for liquid-solid or solid-phase extrac-
tion procedures, e.g., non-polar alkyl phases (from methyl to
octadecyl), polar cyanopropyl and diol types, and also ion-exchan-
ge materials such as aminopropyl and propyl carboxylic acid
phases, to name just a few. Two main advantages of this extrac-
tion approach are of particular interest:
- the speed, enabling more samples to be processed per unit of
  time and
- the use of smaller amounts of organic liquids, mostly water-
  soluble types, which results in reduced safety hazards (ref.
  63).

Liquid-solid extraction methods using bonded silica as a
support started to appear at the beginning of the 1980s. In the
procedures published, octadecylsilica (C_{18}) has been predominantly
used as the support. For the extraction of hydrophilic atenolol
and sotalol, cyanopropyl (CN) silica (ref. 65) and octyl (C_8)
silica supports (ref. 74), respectively, were utilized. The entire
extraction procedure can be divided into four steps, regardless
the type of the support (C_{18}, CN, C_8). A block diagram of the
extraction scheme is depicted in Fig. 3.4.

In the conditioning of the support, as the first step, metha-
nol is used as the organic liquid in most instances. In addition
to water itself, acidic pre-washing (ref. 66), in addition to
alkaline pre-washing (ref. 67), was performed prior to the intro-
duction of the biological sample, mostly plasma. The amount of
sample does not differ from that processed in liquid-liquid ex-
traction and is usually 1 ml. However, it has not been necessary
to adjust the pH to basic values and unchanged plasma can be
extracted directly. Only in two instances, i.e., in the compli-

```
Conditioning solvent
Water                                    COLUMN CONDITIONING

Biological sample                        SAMPLE APPLICATION

Water
Washing solvent                          COLUMN WASHING

Eluting solvent                          ANALYTE ELUTION
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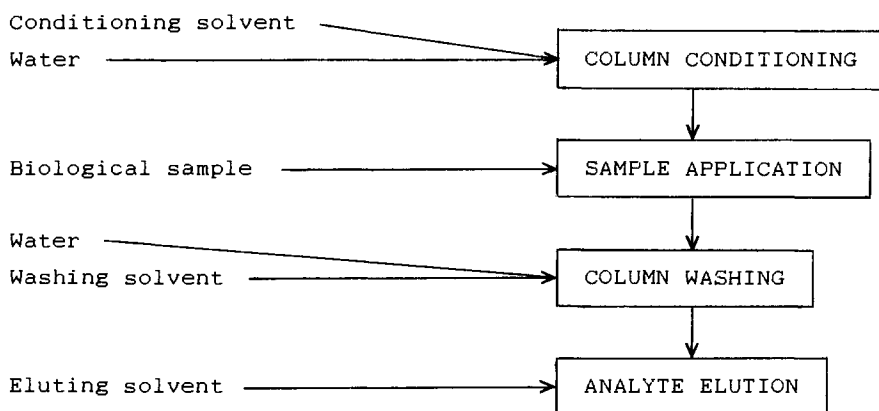
Fig. 3.4. Scheme of the solid-phase extraction of beta-blockers.

cated extraction procedure for timolol, combining two extractions (C_{18}-silica liquid-solid and a subsequent three-step liquid-liquid extraction), and in the extraction of hydrophilic sotalol, before extraction plasma was diluted with phosphate buffer (pH 10.6) (ref. 67) and bicine buffer (pH 9.3 (ref. 74), respectively.

The main task in the third, washing, step is to wash the support free from endogenous compounds and, in doing so, not to lose the majority of the drug. As water itself washes the support only free from biopolymers and hydrophilic substances, the selectivity and sensitivity of analysis can be reduced by the presence of co-extracted lipophilic compounds. With some other groups of drugs this problem was solved by utilizing the different elution abilities of various organic liquids, e.g., by using acetonitrile for the displacement of endogenous compounds, followed by methanol for eluting the purified drugs (refs. 68, 69). With beta-blockers, mixtures of water with methanol and acetonitrile in different ratios have frequently been used. In one instance, pentane was used for the elution of the drug after washing with water and water-methanol (7:3) (ref. 66).

The solvents for the final elution of the drugs from the support can be divided into two categories according to the subsequent processing of the resulting solution. The first category includes methanol, which has been used when evaporation of the eluted solution is intended. This solvent is sufficient for the elution of beta-blockers of different hydrophobicities, e.g.,

propranolol (ref. 70) and nadolol (ref. 66). When the resulting solution from the solid-phase extraction is injected directly into the chromatographic column without further processing, the solvent mixture used for the elution of the retained drug needs to be compatible with the HPLC conditions used. Mostly this applies to mixtures of acetonitrile with hydrochloric acid (ref. 65).

In addition to the previously described manual of off-line solid-phase extraction, the so-called column-switching or on-line extraction procedure has been applied to beta-blockers (ref. 71).

One of the main disadvantages discussed in connection with liquid-solid extraction procedures is the poor comparability of cartridges produced by different manufacturers, as also, from time to time, applies to HPLC columns. In attempting to obtain elution profiles of propranolol using different C_{18} cartridges and methanol-phosphate buffer or methanol alone, the author and his co--workers acquired the following experience. The amount of both solvents needed for quantitative elution of 1 ug of propranolol varied from 2 ml to more than 5 ml, depending on the cartridge used (ref. 72). Therefore, as long as manufacturers are unable to standardize the properties of cartridges, including the properties of silica gel itself, its particle size and shape and the modifying process, "loyalty to one brand" is the only way to prevent this disadvantages. However, batch-to-batch variations from the same manufacturer may also occur.

3.5 FUTURE PERSPECTIVES

The developments in analytical instrumentation aim at simple, reliable, microprocessor-controlled procedures that would permit round-the-clock-processing. Up to the mid 1980s, this development did not cover the conversion of a biological sample in an analytical sample. The introduction of solid-phase extraction as a method of sample pretreatment may also facilitate this last laborious step in bioanalysis. The selectivity of this type of extraction may be further improved in two ways: first, the development of more selective phases for the separation of different drugs, and second, the search for selective solvents and solutions for washing and elution of support-retained drugs.

The combination of automated solid-phase extraction with a sophisticated instrumental analytical method , probably HPLC, seems to be the final goal. This integration would change the

"historical" three-step bioanalytical procedure, i.e., extraction
- measurement - evaluation, into an integrated one-step operation,
with the application of the biological sample as the input and the
final result as the output of the analysis. For the most widely
used beta-blocker, propranolol, its automated solid-phase extrac-
tion using the Varian AASP system has already been described (ref.
73).

REFERENCES

1 E.M. Chait and R.C. Ebersole, Anal. Chem., 53 (1981) 682A-688A.
2 J.W. Black, W.A.M. Duncan and R.G. Shanks, Br. J. Pharmacol.
   Chemother., 25 (1965) 577-591.
3 T. Walle, J. Morrison, K. Walle and E. Conradi, J. Chromatogr.,
   114 (1975) 351-359.
4 Y. Yamamura, K. Uchino, H. Kotaki, S. Isozaki and Y. Saitoh, J.
   Chromatogr., 374 (1986) 311-319.
5 M. Laxer, A.C. Capomacchia and G.E. Hardee, Talanta, 28 (1981)
   973-976.
6 H.Y. Aboul-Enein, M.M.A. Hassan and A.I. Jado, Spectrosc.
   Lett., 16 (1983) 151-158.
7 R.D. Schoenwald and H.S. Huang, J. Pharm. Sci., 72 (1983) 1266-
   1272.
8 J.A. Street and A. Walsh, Eur. J. Pharmacol., 102 (1984) 315-
   324.
9 P. Dallet, J.C. Colleter, E. Audry and J.P. Dubost, Bull. Soc.
   Chim. France, (1980) 294-298.
10 J.M. Criuckshank, Am. Heart J., 100 (1980) 160-178.
11 H. Ishida, M. Sasa and S. Takaori, Jpn. J. Pharmacol., 30
   (1980) 607-617.
12 T. Yamaguchi, C. Ikeda and Y. Sekine, Chem. Pharm. Bull., 34
   (1986) 3362-3369.
13 P. Wang and J. Lien, J. Pharm. Sci., 69 (1980) 662-668.
14 P.B. Woods and M.C. Robinson, J. Pharm. Pharmacol., 33 (1981)
   172-174.
15 P. Hajdu and D. Damm, Arzneim. Forsch. Drug Res., 29 (1979)
   602-606.
16 E.M. Borga, K.M. Piafsky and O.G. Nilsen, Clin. Pharmacol.
   Ther., 22 (1977) 539-543.
17 E. Pike, B. Skuterud, P. Kierulf, D. Fremstad, S.M. Abdel Sayed
   and P.K.M. Lunde, Clin. Pharmacokinet., 6 (1981) 367-374.
18 R.H. Cotham and D. Shand, Clin. Pharmacol. Ther., 18 (1975)
   535-538.
19 S.H.Y. Wong, W.B. White and R. Fernandes, Ther. Drug Monit., 7
   (1985) 358-363.
20 I.B. Kadenatsi and L.E. Kholodov, Klin. Pharm. Zh., 17 (1983)
   1383-1389.
21 J.A.F. de Silva, J. Chromatogr., 340 (1985) 3-30.
22 K.V. Buehring and A. Grabe, J. Chromatogr., 382 (1986) 215-224.
23 W.A. Rietschel and G.A. Thompson, Meth. Find. Exptl. Clin.
   Pharmacol., 5 (1983) 511-525.
24 P. Mahajan, G.D. Grech, R.M. Pearson, E.J. Rodgway and P.
   Turner, Br. J. Clin. Pharmacol., 18 (1984) 849-852.
25 K.M. Karlin, T.J. Zimmerman and G. Nardin, J. Toxicol. Cut.
   Ocular Toxicol., 1 (1982) 155-168
26 C. Schmitt, V.J. Lotti and J.C. Douarec, A.V. Graefes Arch.

Klin. Exp. Ophtalmol., 217 (1981) 167-174.
27 F.M. Gengo, M.A. Zemniak, W.R. Kinkel and W.B. McHugh, J. Pharm. Sci., 73 (1984) 961-963.
28 P.C. Rubin, Scand. J. Clin. Lab. Invest., 44 (1984) 86-89.
29 N.O. Lunell, J. Kulas and A. Rane, Eur. J. Clin. Pharmacol., 28 (1985) 587-599.
30 M.F. O'Hare, G.A. Murnaghan, C.J. Russel, W.J. Leahey, M.P.S. Varma and D.G. McDewit, Br. J. Obstet. Gynaecol., 87 (1980) 814-820.
31 O. Tomčíková, Š. Bezek, M. Ďurišová, V. Faberová, M. Zemánek and T. Trnovec, Biopharm. Drug Dispos., 5 (1984) 153-162.
32 M.R. Gasco, M. Trotta and M.E. Carlotti, Pharm. Acta Helv., (1985) 3-7.
33 T.D. Eller, D.R. Knapp and T. Walle, Anal. Chem., 55 (1983) 1572-1576.
34 S. Sato and I. Yamamoto, J. Immunoassay, 4 (1983) 351-371.
35 M.D. Stern, Clin. Biochem., 17 (1984) 162-165.
36 M. Lo and S. Riegelman, J. Chromatogr., 183 (1980) 213-220.
37 D.D.S. Tang-Liu, S. Liu, J. Richman and R. Weinkam, J. Liq. Chromatogr., 9 (1986) 2237-2252.
38 H.F. Stampfli, C.M. Lai, A. Yacobi and L.Y. Sum, J. Chromatogr., 309 (1984) 203-208.
39 F. Albani, R. Riva and A. Baruzzi, J. Chromatogr., 228 (1982) 362-365.
40 C.L. Rochester, D.E. Gammon, E. Shane and J.P. Bilezikian, Clin. Pharmacol. Ther., 28 (1980) 32-39.
41 P. Haefelfinger, J. Chromatogr., 218 (1981) 73-81.
42 F.O. Mueller, H.K.L. Lundt, P.A. Bromley, J. Torres and O. Vanderbeke, Clin. Pharmacol. Ther., 25 (1979) 528-535.
43 J.P. Remon, P. Gyselinck, R. Synave, R. van Severen and P. Braeckmann, Arch. Pharmacol., 314 (1981) 432-435.
44 J. Gal, P.M. Rhodes, L.M. Nakata and D.C. Bloedow, Res. Commun. Chem. Pathol. Pharmacol., 48 (1985) 255-266.
45 T. Walle, J. Pharm. Sci., 63 (1974) 1885-1891.
46 H. Winkler, W. Ried and B. Lemmer, J. Chromatogr., 228 (1982) 223-234.
47 M.A. Lefebvre, J. Girault and J.B. Fourtillan, J. Liq. Chromatogr., 4 (1981) 483.
48 M. Ervik, K.J. Hoffman and K. Kylberg-Hanssen, Biomed. Mass Spectrom., 8 (1981) 322-326.
49 M. Hata, S. Takahashi, K. Matsubara and Y. Fukui, Jap. J. Legal Med., 43 (1980) 645-650.
50 G.P. Johnston, A.S. Nies and J. Gal, J. Chromatogr., 278 (1983) 204-208.
51 R.B. Gillilan and W.D. Mason, Anal. Lett., 16 (1983) 941-949.
52 M. Ervik, K. Kylberg-Hanssen and P.O. Lagerstöm, J. Chromatogr., 182 (1980) 341-347.
53 R.K. Bhamra, K.J. Thorley, J.K. Vale and D.W. Holt, Therap. Drug Monit., 5 (1983) 313-318.
54 P. Dallet, J.P. Dubost, E. Audry and M.J.C. Colleter, Bull. Soc. Pharm. Bordeaux, 120 (1981) 140-148.
55 M.T. Smith, I. Livingstone, W.D. Hooper, M.J. Eadie and E.J. Triggs, Therap. Drug Monit., 5 (1983) 87-93.
56 L. Bergström, C.G. Johansson, H. Larsson and R. Lenander, J. Pharmacokin. Biopharm., 9 (1981) 419-429.
57 J. McAinsh, in I. Sunshine (Editor), Methodology for Analytical Toxicology, Vol 3, CRC Press, Boca Raton, 1985, pp. 41-46.
58 P.T. Funke, M.F. Malley, E. Ivashkiv and A.I. Cohen, J. Pharm. Sci., 67 (1978) 653-657.
59 E.J. Randinitis, C. Nelson and A.W. Kinkel, J. Chromatogr., 308

(1984) 345-349.

60 M. Schaefer-Korting, N. Bach, H. Knauf and E. Mutschler, Eur.
    J. Clin. Pharmacol., 26 (1984) 125-127.
61 D.B. Jack, S. Dean, M.J. Kendall and S. Laugher, J.
    Chromatogr., 196 (1980) 189-192.
62 L.P. Hackett and L.J. Dusci, J. Forensic Sci., 22 (1977) 379-
    382.
63 R.D. McDowal, J.C. Pearce and G.S. Murkitt, J. Pharm. Biomed.
    Anal., 4 (1986) 9-21.
64 A. Heath, M. Gabrielsson and C.G. Regadh, Br. J. Clin.
    Pharmacol., 15 (1983) 490-492.
65 P.M. Harrison, A.M. Tonkin and A.J. McLean, J. Chromatogr., 339
    (1985) 429-433.
66 R.N. Gupta, R.B. Haynes, A.G. Logan, L.A. MacDonald, R.
    Pickersgill and C. Achber, Clin. Chem., 28 (1983) 1085-1087.
67 J.P. Carlin, R.W. Walker, R.O. Davies, B.T. Fergusson and
    W.J.A. Vandenheuvel, J. Pharm. Sci., 69 (1980) 1111-1115.
68 V. Marko, J. Wijsbeek and R.A. de Zeeuw, J. Pharm. Biomed.
    Anal., 4 (1986) 333-340.
69 V. Marko, Pharmazie, 42 (1987) 387-389.
70 K. Ray, W.G. Trawick and R.E. Mullins, Clin. Chem., 31 (1985)
    131-134.
71 M.W. Lo, B. Silber and S. Riegelman, J. Chromatogr. Sci., 20
    (1982) 126-131.
72 G. Musch and V. Marko, unpublished results.
73 L. Yago, Internat. Lab., 18 (1985) 40-51.
74 M.J. Bartek, M. Vekshtein, M.P. Boarman and D.G. Gallo, J.
    Chromatogr., 421 (1987) 309-318.

Chapter 4

DETERMINATION OF BETA-BLOCKERS BY OPTICAL METHODS

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4.1   INTRODUCTION

Optical properties started to be utilized in quantitative analytical procedures of beta-blockers in 1965. At that time, two research groups, one at ICI, U.K. (J.W. Black, W.A.M. Duncan and R.G. Shanks) and the other at the University of Frankfurt, FRG (K. Stock and E. Westermann), published papers describing the spectrofluorometric determination of pronethalol, propranolol (ref. 1) and toliprolol (ref. 2).

Although during the past two decades eight further methods have been developed and used for the determination of beta-blockers in biological material (spectrofluorometry, gas--liquid chromatography, thin-layer chromatography, high-performance liquid chromatography, radioimmunoassay, enzyme immunoassay, fluorescence immunoassay, radioreceptor assay), spectrofluorometry has remained until today. For comparison with recent methods, the main features of the first two introduced methods are shown in Table 4.1.

4.2   SPECTROPHOTOMETRIC PROPERTIES OF BETA-BLOCKERS

As optical methods for the determination of beta-blockers are based on a knowledge of the optical properties of the drugs, part of this chapter will be devoted to these properties of beta--blockers.

TABLE 4.1

Main features of spectrofluorometric methods according to Black et al. (ref. 1) and
Stock and Westerman (ref. 2).

	Method	
	Black et al.	Stock and Westermann
Drugs	pronethanol, propranolol	toliprolol (Kö 592)
Biological material	blood, tissues	blood, tissues, urine
pH adjustment	NaOH, 0.1 mol l^{-1}	NaOH, 5 mol l^{-1}
Solvent - extraction	1% ethanol in heptane	benzene
- back-extraction and measurement	HCl, 8.1 mol l^{-1}	HCl, 0.1 mol l^{-1}
Recovery (blood)	80%	79%
λ (nm)	280/350	275/312
Sensitivity (ng ml^{-1})	10	60

TABLE 4.2

Characteristic UV absorption data for 20 beta-blockers (refs. 4-9)

Drug	λ(nm)/ $\varepsilon.10^3$ (l mol^{-1} cm^{-1})			
	methanol	water	HCl (0.1 mol l^{-1})	NaOH (0.1 mol l^{-1})
Acebutolol	328/ 2.58	320/ 2.54	320/ 2.54	
	235/26.40	234/21.74	234/22.04	
Alprenolol	277/ 1.65	276/ 1.59	276/ 1.52	276/ 1.77
	271/ 1.84	271/ 1.78	270/ 1.71	
Atenolol	275/ 1.43	275/ 2.1	274/ 1.26	275/ 1.30
	227/10		225/ 9.29	
Bunitrolol	291/ 3.95		291/ 4.05	294/ 4.24
	231/ 9.18		232/ 9.47	232/ 9.69
Bupranolol	283/ 6.24		282/ 1.54	283/ 1.57
	276/ 1.79		275/ 1.66	276/ 1.72
Labetalol	305/ 3.49		303/ 3.14	333/ 5.29
				246/ 8.8
Metipranolol	279/ 1.64		278/ 1.36	
Metoprolol	282/ 2.62	280/ 2.94[a]	281/22.31[a]	275/ 1.73
	276/ 3.11	274/ 3.60	274/ 2.83	
	223/21.5	223/23.4	224/19.5	

Nifenalol	266/10.37		273/ 9.95	278/ 9.43
Oxprenolol	273/ 2.33	273/ 2.32	272/ 2.33	272/ 2.41
Practolol	248/16.24	250/10.08	243/12.09	244/12.14
Sotalol	274/ 5.55		269/ 4.20	250/15.02
	232/14.56			
Toliprolol	279/ 1.36	278/ 1.44	278/ 1.18	
	272/ 1.43	271/ 1.48	272/ 1/32	
	218/ 7.34			
Penbutolol	278/ 2.06	276/ 1.81	270/ -[b]	
	272/ 2.24	271/ 2.07	215/ -[b]	
Nadolol	279/ 1.13		277/ 1.0	278/ 1.03
	271/ 1.10		270/ 1.12	271/ 1.06
Propranolol	319/ 1.95	319/ 2.01	319/ 2.01	319/ 2.22
	289/ 6.12	289/ 5.80	288/ 5.80	291/ 5.89
Carazolol	332/ 6.24	331/ 5.07	331/ 5.28	331/ 5.37
	285/14.77	284/11.58	284/12.23	284/12.23
	242/52.50	240/45.66	240/48.34	240/47.74

TABLE 4.2 (continued)

Drug	$\lambda(nm)/ \varepsilon.10^3$ $(1\ mol^{-1}\ cm^{-1})$			
	methanol	water	HCl (0.1 mol l^{-1})	NaOH (0.1 mol l^{-1})
Mepindolol	287/ 9.30	267/18.90	267/18.90	267/19.10
	267/20.40			
Pindolol	288/ 4.20	287/ 4.63	287/ 3.80	285/ 3.92
	265/ 7.95	267/ 7.59	264/ 7.25	262/ 7.42
Timolol	299/ 9.07		295/ 8.54	296/ 8.92

[a] shoulder.

[b] not given.
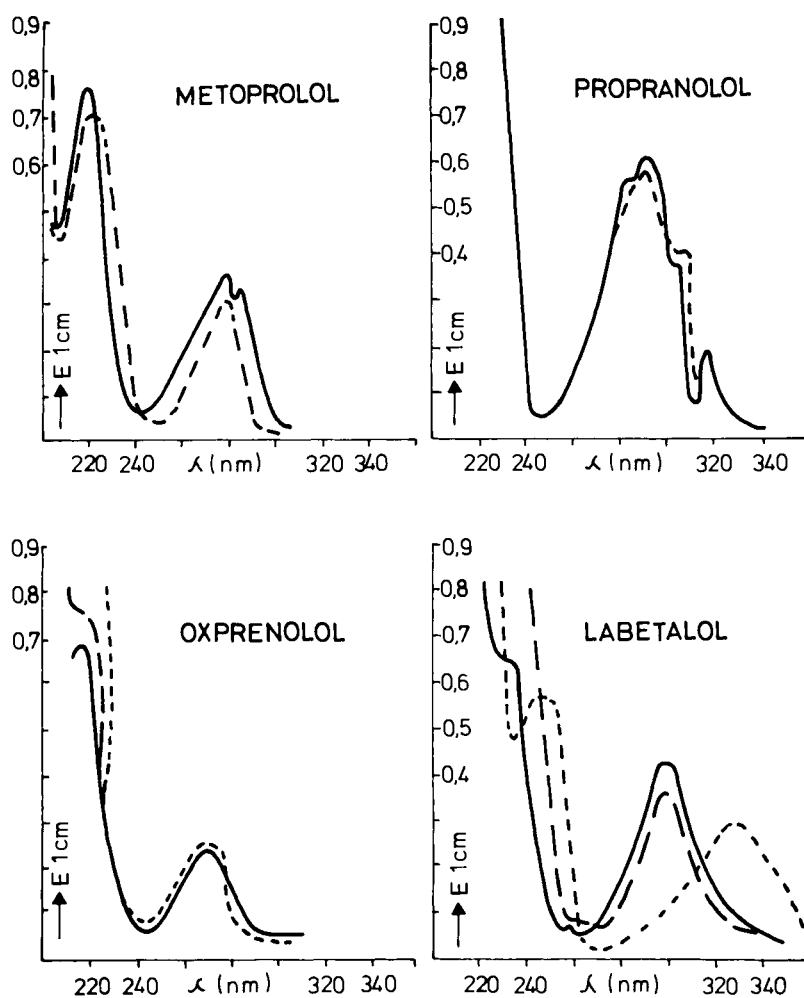
Fig 4.1. UV spectra of metoprolol, oxprenolol, propranolol and labetalol in methanol (————), HCl (0.1 mol l^{-1}) (— — —) and NaOH (0.1 mol l^{-1}) (------).

104

weakly fluorescent. Its fluorescence can be influenced by the type
and position of substituents. Thus, fluorescence can be increased
by an electrophilic group and by substitution at ortho and para
positions; the meta position of two substituents represses the
fluorescence of aromatic compounds (ref. 10). This is only a
general rule, as seen with the beta-blockers penbutolol, alpreno-
lol and oxprenolol. They are ortho-substituted in the oxopropanol-
amine side-chain. However, whereas the first two possess intrinsic
fluorescence (refs. 8, 9), oxprenolol does not (ref. 11).

Unlike the UV absorption of beta-blockers, which is relative-
ly well described, much less attention has been paid to the fluo-
rescence of these drugs and to the influence of various condi-
tions on it. One of the few excitation/emission spectra in the
literarture is that of atenolol (Fig. 4.2).
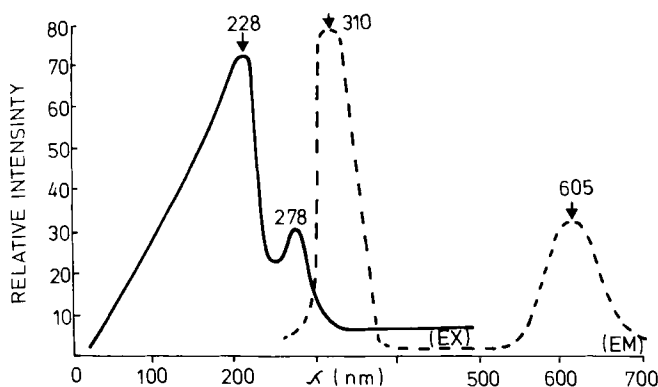
Fig. 4.2. Excitation and emission spectra of atenolol (ref. 12).
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The pH dependence of the fluorescence intensity of beta-
-blockers was first described by Potter for propranolol in 1967
(ref. 13). He obtained the maximum fluorescence intensity of the
drug at pH 3.5. The experience of Marko with the pH dependence of
the fluorescence intensities of different beta-blockers showed
the optimal pH for propranolol to be approximately one unit higher
(between 4 and 5), as it was also for other beta-blockers, namely
alprenolol, atenolol, exaprolol, metaprolol and toliprolol (ref.
9). The pH dependence of four of these drugs, alprenolol, ateno-

lol, metoprolol and propranolol, are shown in Fig. 4.3.
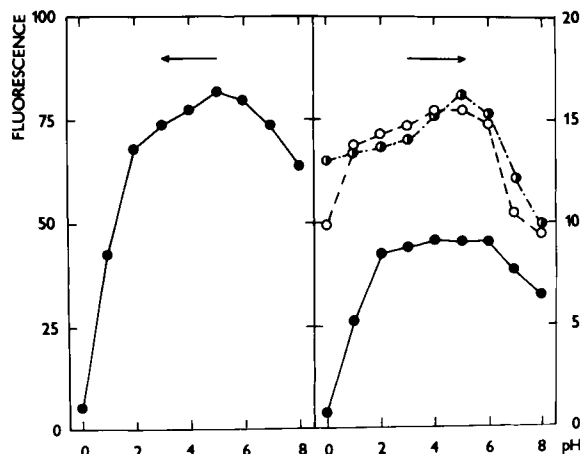
Fig 4.3. pH dpendences of the fluorescence intensities of propra-
nolol (left) and alprenolol (o- - - -o), atenolol (●————●) and
metoprolol (●—·— ·●) (right). The excitation/emission maxima were
305/342 nm for propranolol, 275/294 nm for alprenolol, 280/295 nm
for atenolol and 279/297 nm for metoprolol (ref. 9).

4.3 OPTICAL METHODS

Optical methods for the determination of beta-blockers in
biological material were developed for 17 drugs of this group,
namely acebutolol, atenolol, bevantolol, carteolol, celiprolol,
labetalol, metoprolol, nadolol, penbutolol, pindolol, practolol,
pronethanol, propranolol, sotalol, talindolol , toliprolol and SL-
-75212. For these methods, measurement of both transmitted and
emitted light was utilized.

4.3.1 Spectrophotometry and colorimetry

As shown in Table 4.2, the molar absorption coefficients of
beta-blockers are in the range 10^3 - 10^4. This means that the
lowest concentrations of the drugs which can give a measureable
absorption (e.g., tenths of a unit) have to be in the ug ml^{-1}
range. This range is, e.g., in the case of propranolol, two orders
of magnitude higher than the therapeutic concentration of the
drug. Therefore, the direct measurement of UV absorbance could be
used for the determination of beta-blockers in body fluids only
for toxicological purposes (ref. 14). In materials other than body

fluids, spectrophotometry was used, e.g., for the determination of bevantolol in animal feeds (ref. 15).

For obtaining absorbance at a level that allows the determination of therapeutic concentrations of beta-blockers, coupling of strong chromophores to molecule of these drugs had to be performed. For this purpose, Bratton-Marshall reagent, N-(1-naphthyl)-ethylenediamine hydrochloride, was used with four beta-blockers, acebutolol (ref. 16), celiprolol (ref. 17), practolol (ref. 18) and talinolol (ref. 19). The use of this reagent required the presence of an aromatic -NH-CO- group in the structure of the beta-blocker, as the first step of the reaction was acid hydrolysis of this group to the primary aromatic amine which, after diazotation, reacted with N-(1-naphthyl)ethylenediamine to give the azo dye. The scheme of this three-step reaction is shown in Fig. 4.4. By using Bratton-Marshall reagent the sensitivity of spectrophotometric determination was enhanced to the levels of tens of nanograms per millilitre of body fluid. In principle, this allowed therapeutic concentrations of the four drugs to be determined. The main disadvantage of this method was its lack of specificity, as all metabolites that could be diazotized were co-determined. This is shown for acebutolol and its main metabolite in Fig. 4.4. Colorimetry was abandoned as a method for the determination of beta-blockers in biological material in the late 1970s.

4.3.2 Spectrofluorometry

As indicated in the Introduction to the book, spectrofluorometry was the first method to be used for the determination of beta-blockers in biological material. Most beta-blockers possess native fluorescence and, therefore measurement can be provided directly, without prior derivatization with fluorescent reagents. This was performed with nine beta-blockers, i.e., atenolol, labetalol, metoprolol, penbutolol, pronethanol, propranolol, sotalol, toliprolol and SL-75212 (ref. 20).

If the drug has no native fluorescence, or if its intensity is not sufficient for the purpose, less or more complicated derivatization is required (acebutolol, nadolol, pindolol and practolol). With carteolol, fluorescence is induced by irradiation.

(i) Direct measurement. Condition for the determination of atenolol, labetalol, metoprolol, penbutolol and sotalol, shown in Table 4.3, can serve as examples of the methods used for the

$R^1-NH-CH_2-CH-CH_2-O-$ [ring] $-NH-R^3$
$\overset{\displaystyle |}{OH}$ $\quad R^2$

$\xrightarrow{HCl}$

$R^1-NH-CH_2-CH-CH_2-O-$ [ring] $-NH_2$
$\overset{\displaystyle |}{OH}$ $\quad R^2$

$\longrightarrow$

1. NO_2^-

$\xrightarrow{\hspace{3cm}}$

2. [naphthalene] $-NH-CH_2-CH_2-NH_2$

$R^1-NH-CH_2-CH-CH_2-O-$ [ring] $-N=N-$ [naphthalene]
$\overset{\displaystyle |}{OH}$ $\quad R^2$ $\quad\quad -NH-CH_2-CH_2-NH_2$
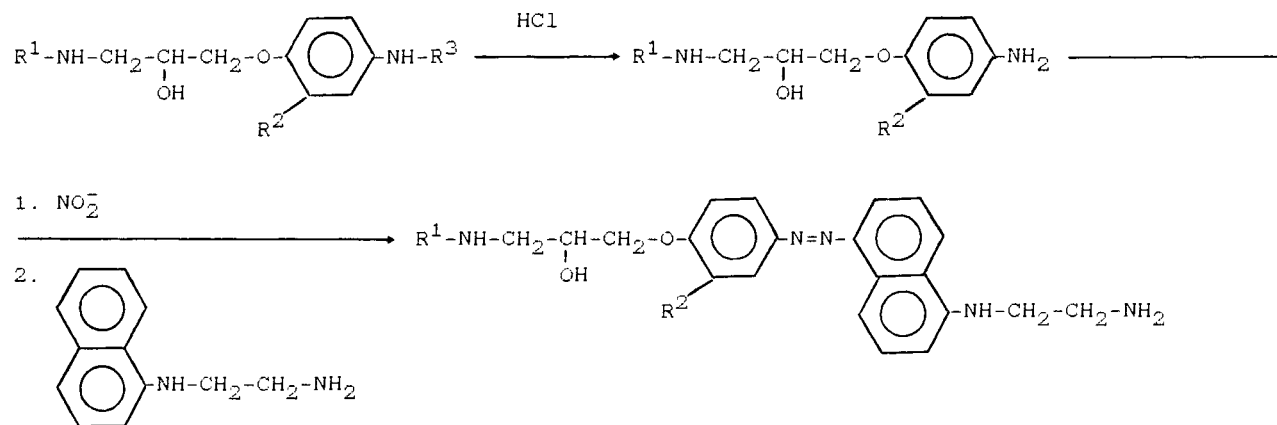
	R^1	R^2	R^3	$\lambda(nm)$	Ref.
Acebutolol	$CH(CH_3)_2$	CH_3CO	C_3H_7CO		16
Acebutolol metabolite	$CH(CH_3)_2$	CH_3CO	CH_3CO		16
Celiprolol	$C(CH_3)_3$	CH_3CO	$(C_2H_5)_2NCO$	565	17
Practolol	$CH(CH_3)_2$	H	CH_3CO	580	18
Talinolol	$C(CH_3)_3$	H	$C_6H_{11}NHCO$	590	19

Fig. 4.4. Scheme of the derivatization of beta-blockers using Bratton-Marshall reagent.

spectrofluorometric determination of beta-blockers. In all instances the same extraction procedure, i.e., extraction into an organic phase (after alkalinization of the sample) and back--extraction into hydrochloric acid, was performed. Hydrochloric acid served also as the measuring solvent. In one instance, XAD-2 was used for liquid-solid extraction from urine (ref. 21). The limits of determination were, in principle, sufficient for the practical utilization of the methods.

So far as we know, thirteen methods have been described for the spectrofluorometric determination of the most used (and the most frequently determined) beta-blocker, propranolol. Most of them do not differ substantially from the method of Black et al. (ref. 1), described at the beginning of this chapter. The most favourite extraction mixture is 1.5% isoamyl alcohol in hexane, and HCl (0.01 - 0.1 mol l^{-1}) is the favourite back-extraction and measuring liquid. The limits of determination with these methods are approximately 10 ng ml^{-1}.

Ambler et al. (ref. 25) showed that replacement of hydrochloric acid with weaker acids, e.g., citric acid and its mixture with ethylene glycol, resulted in improved sensitivity. They achieved the determination of 5 ng ml^{-1} of propranolol in plasma. Rao et al. (ref. 26) used a mixture of acetic acid (5.10^{-3} mol l^{-1})and ethylene glycol and reported a limit of determination of 2.5 ng ml^{-1}.

A different approach to the measurement of plasma concentrations of propranolol was used by Capomacchia and Vallner (ref. 27). They diluted plasma samples with dimethyl sulphoxide - water (1:2, v/v) and, by direct measurement of the fluorescence of this mixture at excitation/emission maxima of 317/340 nm, determined the concentration of the "total propranolol", i.e., propranolol, its acidic metabolites and glucuronides. After removing propranolol from the mixture by extracting it with diethyl ether or ethyl acetate, the concentartion of the parent drug was expressed as the difference in the fluorescence intensity of the aqueous layer before and after extraction. The lowest detectable concentration was 10 ng ml^{-1}.

(ii) Induced fluorescence. Two types of chemical transformation resulting in fluorescent species were described, i.e., formation of fluorescent derivatives with appropriate coupling agents and direct action by the use of UV light. In the determination of pindolol, combination of the coupling method with irradiation was

TABLE 4.3.

Conditions for the spectrofluorometric determination of five beta-blockers

Drug	Biological fluid[a]	Solvents		λ (nm)	Sensitivity (ng ml^{-1})	Ref.
		extraction	measuring			
Atenolol	P, U	dichloromethane:BuOH 95:5	HCl 0.01 mol l^{-1}	280/305	10	21
Labetalol	P, U	chloroform (XAD-2)	HCl 1 mol l^{-1}	334/412	20	22
Metoprolol	S	benzene	HCl 0.1 mol l^{-1}	275/305	$-$[b]	23
Penbutolol	P, U	heptane:isoamyl-OH 98.5:1.5	HCl 0.1 mol l^{-1}	280/300	5	8
Sotalol	P	ethyl acetate	HCl 3 mol l^{-1}	234/309	100	24

[a] P = plasma, U = urine, S = serum.
[b] not given.

used.

Three derivatization methods were used for coupling beta-blockers with a fluorophore, i.e., reduction of amino group and diazotation and coupling with -nitrosonaphthol (acebutolol and practolol), reaction with o-phthalaldehyde (pindolol) and a two--step reaction involving oxidation and coupling with o-phenylene-diamine (nadolol).

The first method has the same principle (and the same dis-advantage) as that using Bratton-Marshall reagent in spectrophoto-metry, discussed in Section 4.3.1. Moreover, the sensitivity of this non-specific determination is not very high compared with other spectrofluorometric methods (hundreds of nanograms per millilitre) (refs. 28, 29). Lack of specificity has not been reported in the derivatization of pindolol with o-phthalaldehyde. The reaction occured in hydrochloric acid (0.1 mol l^{-1}) and it was activated either by heating (ref. 30) or by UV light (ref. 31). The product had excitation/emmision maxima at 390/440 nm and an approximately 10^4 times higher fluorescence intensity than the parent drug. The latter activation method led to an approximately ten times lower limit of determination compared with thermal activation (2 ng ml^{-1} plasma).

The derivatization of nadolol consists of two steps. After extraction of the drug from plasma (n-butyl acetate) or urine (diethyl ether), oxidation with periodic acid was performed. The resulting dialdehyde reacted with an aromatic diamine (o-phenyle-nediamine) to yield the Schiff base with excitation/emission maxi-ma of 305/445 nm. The sensitivity of this method was at the level of nanograms per millilitre (ref. 32).

Enhancement of fluorescence by UV irradiation was used in the determination of carteolol (ref. 33). After a three-step extrac-tion procedure (chloroform - sulphuric acid - chloroform), the organic phase was evaporated, the extract was dissolved in hydro-chloric acid (1 mol l^{-1}) and the solution was irradiated for 80 min using a tungsten lamp. Subsequently, the fluorescence of the reaction product, i.e., the 3,4-dehydro derivative of carteolol (Fig. 4.5), was measured, with emission at 460 nm following exci-tation at 310 nm. If hydrobromic acid instead of hydrochloric acid is used as the reaction medium, no fluorescence occurs. Down to 100 ng ml^{-1} of carteolol in plasma could be measured by this method.
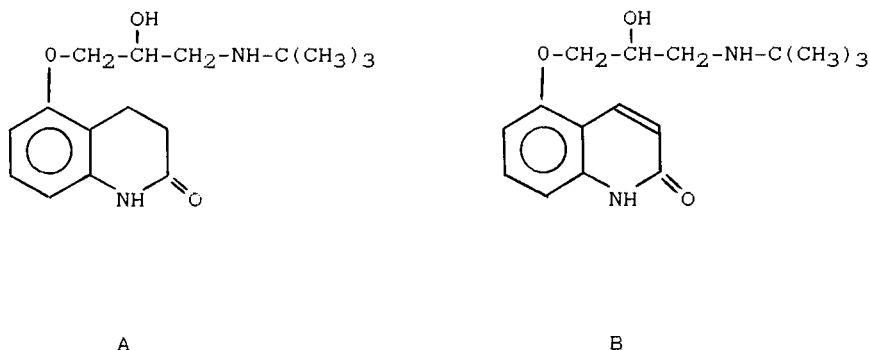
Fig. 4.5. (A) Carteolol and (B) its 3,4-dehydro derivative.

4.4 VALIDITY OF SPECTROFLUOROMETRY

Two shortcomings have been reported for the spectrofluoromet-
ric determination of beta-blockers: lack of sensitivity and lack
of specificity. As shown in this chapter, there are optical me-
thods that are non-selective in principle (diazotation of acebuto-
lol) and those which have low sensitivity. However, in our opi-
nion, this problem is more complicated than is implied in the
literature.

Concerning the first disadvantage, literature data are some-
times controversial. On the one hand there are notes, mostly in
introductions to chromatographic papers, on high and variable
blank values of spectrofluorometric determinations of some beta-
-blockers (refs. 34, 35) and on the low sensitivity that result
from them. In some instances, a paper by Chidsey et al. (ref. 36)
is to referred to, in which the authors mentioned their problems
with the accurate quantification of many samples in which propra-
nolol was found at a concentration of less than 30 ng ml^{-1}. On the
other hand, the limits of determination published for various
spectrofluorometric and gas-liquid chromatographic methods are
comparable, as shown for propranolol and pindolol as examples in
Table 4.4.

Concerning the second shortcoming, only studies on the effect
of propranolol metabolites on its fluorometric assay in plasma
have been performed. Pritchard et al. (ref. 39) divided proprano-

TABLE 4.4.

Limits of determination of some spectrofluorometric (SFM) and gas-
-liquid chromatographic (GLC) methods for propranolol and pindo-
lol

Method	Limit of determination (ng ml^{-1})		
	Propranolol		Pindolol
SFM	2.5 (ref. 26)		2 (ref. 31)
GLC - packed column	5 (ref. 34)		1 (ref. 35)
capillary column	1 (ref. 37)		0.5 (ref. 38)

lol metabolites into two groups. The metabolites of the first
group, i.e., N-desisopropylpropranolol and propranolol glycol,
have comparable, or at least detectable, fluorescence intensities
at the excitation/emission maxima of propranolol, but their con-
centrations in plasma are too low to affect the determination of
propranolol. The metabolites of the second group, i.e., 4-hydroxy-
propranolol, -naphthol and dihydroxynaphthalene, have fluorescen-
ce maxima sufficiently far from that of propranolol and therefore
also do not have an adverse effect.

The best way to assess the validity of the spectrofluoromet-
ric method is to make a direct comparison with another method or
methods used for the determination of the same drug in the same
sample. We shall discuss here four comparisons, all involving
chromatographic methods. Two of the papers considered compare
spectrofluorometric and gas-liquid chromatographic methods (for
the determination of pindolol and nadolol), one compares spectro-
fluorometry with thin-layer chromatography (for talinolol) and one
spectrofluorometry with high-performance liquid chromatography
(for propranolol).

Guerret et al (ref. 40) used two methods, capillary gas-
-liquid chromatography after extraction and derivatization with
trifluoroacetylimidazole (ref. 38) and spectrofluorometry invol-
ving condensation of the drug with o-phthalaldehyde (ref. 30) for
the determination of the absolute oral bioavailability of pindolol
in six healthy volunteers. The study showed good agreement of the
results obtained by the two methods, as can be seen from Fig. 4.6,
and thus confirmed the specificity of the spectrofluorometric
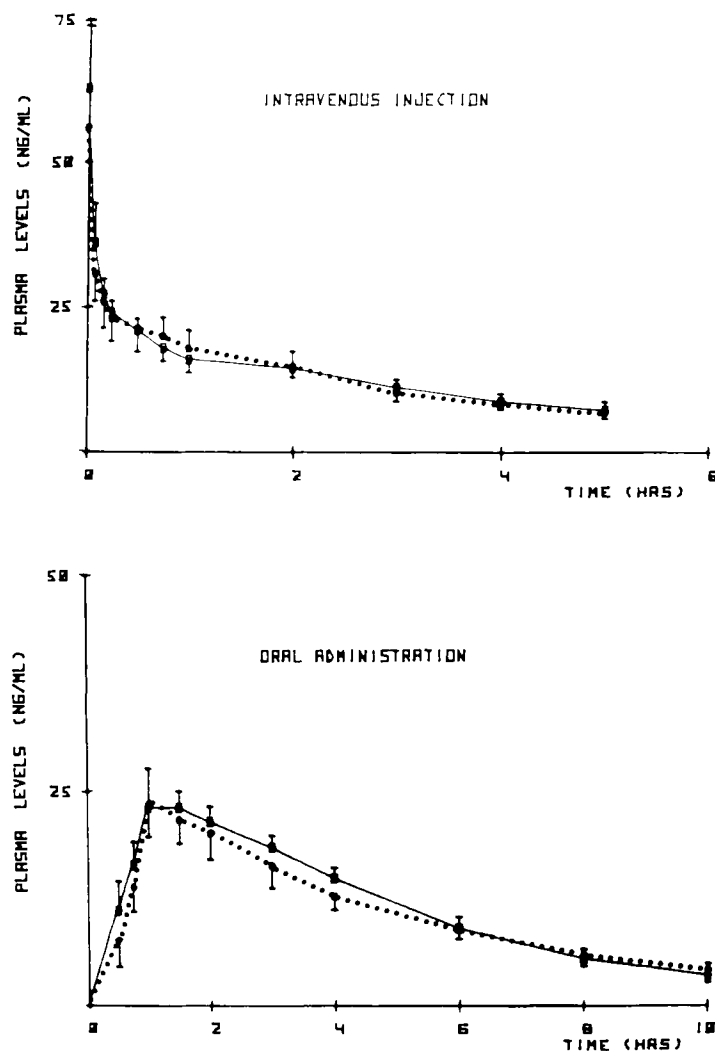
Fig. 4.6. Mean plasma concentration-time curves after administra-
tion of pindolol, 3 mg i.v. and 5 mg p.o.; mean ± SEM; (————)
fluorometric assay; (·········) gas chromatographic assay (ref. 40).
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method for determination of pindolol. The correlation coefficient
of the gas-liquid chromatography-spectrofluorometry dependence was
0.931.

114

Funke et al. (ref. 41) compared gas chromatography-mass
spectrometry and spectrofluorometry of another beta-blocker,
nadolol, as and addition to the development a gas chromatographic
method. For this purpose, duplicate specimens from patients
receiving an oral dose of nadolol were randomly sampled and coded
for analysis by both the fluorometric method and selective ion
monitoring. The results were presented as the difference between
the two assays plotted against the average of the assay results
(Fig. 4.7). Although there was a systematic trend indicating that
greater concentrations were obtained by fluorometry than by
selective ion monitoring, this was not significant enough to
prevent the authors from concluding that the spectrofluorometric
data represented an accurate measure of nadolol levels in blood.
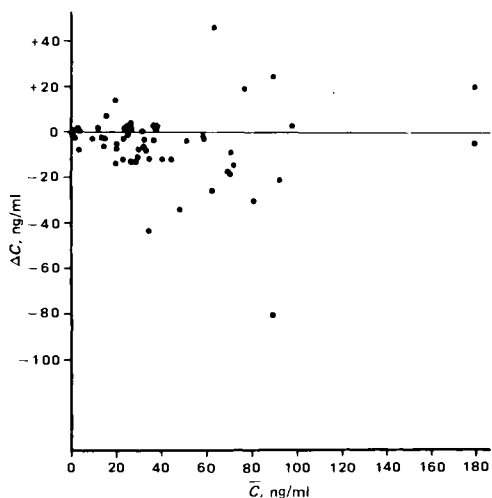
Fig. 4.7. Plott of difference between nadolol assays, ΔC (GLC-mass
spectrometric - fluorometric), versus the average of the assays, $\overline{C}$
(ref. 41). Reproduced with permission of the copyright owner, the
American Pharmaceutical Association.

Comparison of spectrofluorometry with thin-layer chromatography was performed during the development of a TLC screening method for monitoring the drug compliance of talinolol-receiving patients (ref. 42). The dependence of the data obtained by the semi-quantitative thin-layer chromatographic method and the spectrofluorometric method is shown in Fig. 4.8. The correlation was described as
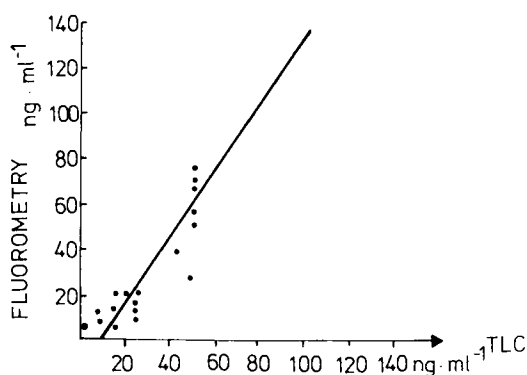
Fig. 4.8. Correlation between a semi-quantitative thin-layer chromatographic and spectrofluorometric methods for the determination of talinolol in urine (ref. 42). Reproduced with permission of the copyright owner.

linear with a correlation coefficient of 0.95. Nevertheless, on careful examination slight non-linearity in the low-concentration area can be detected.

In all three instances above there was a good agreement between the spectrofluorometric method and chromatographic methods. In both GLC comparisons this agreement was also achieved at low concentrations of the drugs. However, none of the papers did state whether patients· own blank plasma samples were available in the spectrofluorometric determinations.

A comparison of high-performance liquid chromatographic and spectrofluorometric methods for the determination of propranolol published by Wood et al. (ref. 43) clearly showed the necessity to incorporate blank plasma samples. When plasma blanks from the subjects were available, the comparison of HPLC and fluorometry

116

correlated well. On the other hand, when plasma blanks were not
available, no correlation was obtained (Fig. 4.9). Differences
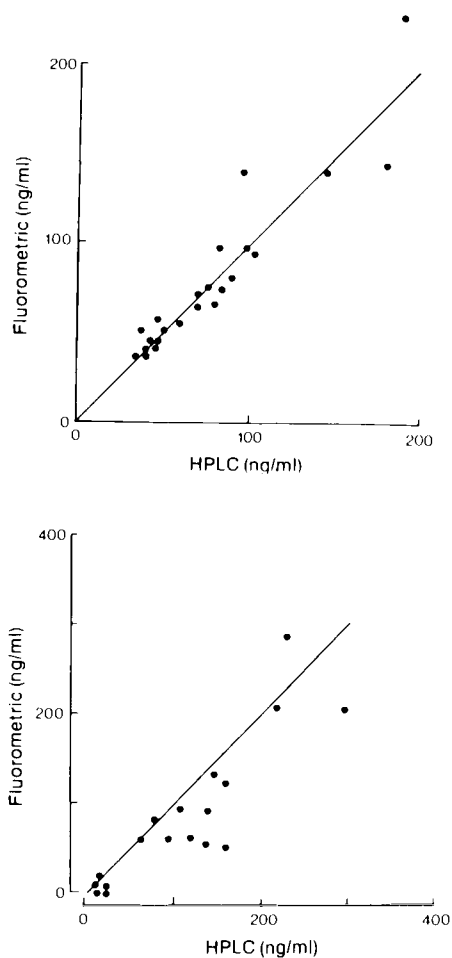
Fig. 4.9. (A) Comparison of HPLC and fluorometric methods for the
determination of propranolol in samples (n=22) for which blank
plasma from the subjects was available. The best fit of the data
is given by y = 1.238 + 1.004x (r=0.93) and was not significantly
different from the line of identity.

(B) Comparison between HPLC and fluorometric assays of
propranolol in random clinical samples (n=17). The best fit was
given by y = 21.470 + 0.584 (r=0.79) and was significantly diffe-
rent from the line of identity (ref. 43). Reproduced with per-
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were observable whatever the concentration measured, i.e., at both low and high concentrations.

What conclusions can be drawn from these comparisons? Let us consider the criteria used when evaluating methods of bioanalysis, sensitivity, specificity and robustness of the method (ref. 44).

Comparison of the limits of determinations of properly used spectrofluorometric and chromatographic methods does no yield any great difference. Similarly, in direct comparisons the spectro-fluorometric method was able to measure the same concentrations as chromatographic methods. With pindolol, propranolol and nadolol, the lowest concentrations measured by the two methods compared were in the range of nanograms per millilitre.

The co-determination of metabolites, one of the indices of non-specificity of a method, was excluded, as demonstrated either by comparing the optical and pharmacokinetic properties of metabo-lites and the parent drug (propranolol), or by the good correla-tion of the data obtained by spectrofluorometry and chromatography (talinolol, pindolol, nadolol, propranolol). The serious problem with spectrofluorometric measurements is the variable level of the plasma blank. If it is not known and hence not considered in the determination , it can cause biased results. This fact handicaps the spectrofluorometric method mainly in routine clinical studies when the patient s own plasma or serum blank is frequently not available.

The main advantage of the spectrofluorometric method over chromatographic methods is its simplicity, i.e., it is possible to process more samples, less trained personnel are required for this processing and cheaper instruments are available for fluorescence measurements than for chromatographic analysis. This simplicity is not affected by sample preparation, as there is no great differen-ce between pre-spectrofluorometric and pre-chromatographic sample handling (see Chapter 3).

In addition to comparisons of spectrofluorometric and chroma-tographic methods, one paper was devoted to the comparison of spectrofluorometry with one of the competitive binding assays, i.e., fluoroimmunoassay (ref. 45). As can be seen from Fig. 4.10, also in this instance good agreement between the two assays was obtained (r = 0.99). However, it was not stated whether plasma blanks were or were not available.

118
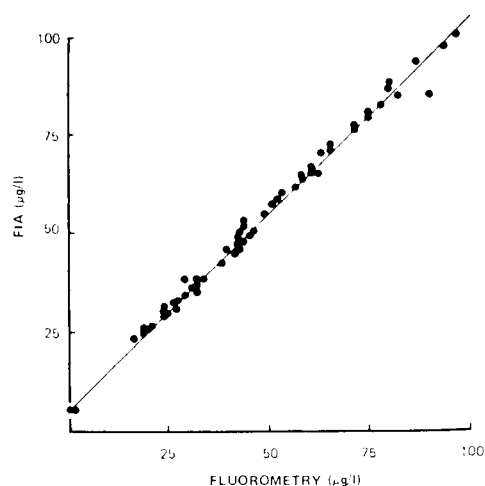
Fig. 4.10. Correlation between propranolol levels in patients'
serum or plasma specimens determined by fluoroimmunoassay (y) and
spectrofluorometry (x). Reproduced with permission of the copy-
right owner.

The sensitivity of competitive binding assays is the best of
all the methods used in the bioanalysis of beta-blockers and is
better than that of spectrofluorometry. Also, a satisfactorily
high specificity of immunoanalytical methods towards metabolites
has been achieved. As endogenous compounds have a minor influence
on the determination of the drugs, the concentration of beta-
-blockers can be measured in unprocessed plasma or serum. The
great disadvantage of competitive binding assays for beta-blockers
is the need to prepare a "tool" for this determination, i.e.,
antibodies or receptors, as no commercial kit is available.

On balance, then, spectrofluorometry, if properly used, still
has a place among assays for the determination of beta-blockers in
biological material. Its future is secured mainly by its major
advantage, i.e., simplicity. Along with the development of more
sophisticated chromatographic instruments and the introduction of
commercially available immunoassay kits, the role of spectro-
fluorometry will continue to decrease.

REFERENCES

1 J.W. Black, W.A.M. Duncan and R.G. Shanks, Brit. J. Pharm. Chemother., 25 (1965) 577-591.
2 K. Stock and E. Westermann, Biochem. Pharmacol., 14 (1965) 227-236.
3 E.L. Wehry, in Guibault (Editor), Practical Fluorescence, Marcel Dekker, New York, 1973.
4 H.W. Dibbern and E. Wirbitzki, UV and IR Spectra of Some Important Drugs, Editio Cantor, Aulendorf, 1978.
5 M.E. Mohamed, M.S. Tawakkol and H.Y. Aboul-Enein, Spectrosc. Lett., 15 (1982) 609-621.
6 S.O. Jansson and S. Johansson, J. Chromatogr., 242 (1982) 41-50.
7 J.R. Luch, Anal. Profiles Drug Substances, 12 (1978) 325-356.
8 P. Hajdú and D. Damm, Arzneim. Forsch. Drug Res., 29 (1979) 602-606.
9 V. Marko, unpublished results.
10 G. Guibault, Practical Fluorescence, Marcel Dekker, New York, 1973.
11 M. Schaefer and E. Mutschler, J. Chromatogr., 164 (1979) 247-252.
12 C. Verghese, A. McLeod and D. Shand, J. Chromatogr., 275 (1983) 367-375.
13 L.T. Potter, J. Pharmacol. Exp. Ther., 155 (1967) 91-100.
14 R. Swaw, in I. Sunshine (Editor), Methodology in Analytical Toxicology, CRC Press, Cleveland, 1975, pp. 328-331.
15 C.H. Spurlock and H.G. Schneider, J. Assoc. Off. Anal. Chem., 67 (1984) 321-324.
16 C.M. Kaye, C.R. Kumana, M. Leighton and P. Turner, Clin. Pharmacol. Ther., 19 (1976) 416-420.
17 F. Takacs and D. Hippmann, Arzneim. Forsch. Drug Res., 33 (1983) 5-8.
18 J.D. Fitzgerald and B. Scales, Int. Z. Klin. Pharmakol. Ther. Toxicol., 1 (1968) 467-474.
19 H. Nuernberger, Pharmazie, 30 (1975) 671-673.
20 B.J. Davis and P. Turner, Brit. J. Clin. Pharmacol., 8 (1979) 405P.
21 B. Flouvat, M. Bazin, M. Lucsko, A. Roux and J. Guedon, Ann. Biol. Clin., 36 (1978) 339-346.
22 L.E. Martin, P. Carey and R. Bland, Methodol. Surv. Biochem., 7 (1978) 227-242.
23 M. Počtová and B. Kakáč, Ceskoslov. Farm., 34 (1985) 222-225.
24 H.C. Brown, S.G. Carruthers, J.G. Kelly, D.G. McDewitt and R.G. Shanks, Eur. J. Clin. Pharmacol., 9 (1976) 367-372.
25 P.K. Ambler, B.N. Singh and M. Lever, Clin. Chim. Acta, 54 (1974) 373-375.
26 P.S. Rao, L.C. Quesada and H.S. Mueller, Clin. Chim. Acta, 88 (1978) 355-361.
27 A.C. Capomacchia and J.J. Vallner, J. Pharm. Sci., 69 (1980) 1463-1465.
28 G. Bodem and C.A. Chidsey, Clin. Chem., 18 (1972) 363-365.
29 A. Roux, B. Flouvat and P. Delaveau, Anal. Biol. Clin., 33 (1975) 281-288.
30 W.L. Pacha, Experientia, 25 (1969) 802-803.
31 R. Gugler, L. Kreis and J.H. Dengler, Arzneim. Forsch. Drug Res., 25 (1975) 1067-1072.
32 E. Ivashkiv, J. Pharm. Sci., 66 (1977) 1168-1172.
33 M. Kido, H. Mori, S. Masuda, H. Kohri and K. Nakagawa, Yakugaku

Zasshi, 97 (1977) 1-4.
34 E. di Salle, K.M. Baker, S.R. Bareggi, W.D. Watkins, C.A. Chidsey, A. Frigerio and P.L. Morselli, J. Chromatogr., 84 (1973) 347-353.
35 M. Guerret, J. Chromatogr., 221 (1980) 387-392.
36 C.A. Chidsey, P. Morselli, G. Bianchetti, A. Morganti, G. Leonetti and A. Zanchetti, Circulation, 52 (1975) 313-318.
37 A.G. de Broer, D.D. Breimer and J.M. Gubbens-Stibbe, Pharm. Weekbl., Sci. Ed., 2 (1980) 101-105.
38 M. Guerret, D. Lavene and J.R. Kiechel, J. Pharm. Sci., 69 (1980) 1191-1193.
39 J.F. Pritchard, D.W. Schneck, W.J. Racz and A.H. Hayes, Clin. Biochem., 11 (1978) 121-125.
40 M. Guerret, G. Cheymol, J.P. Aybry, A. Cheymol, D. Lavene and J.R. Kiechel, Eur. J. Clin. Pharmacol., 25 (1983) 357-359.
41 P.T. Funke, M.F. Malley, E. Ivashkiv and A.I. Cohen, J. Pharm. Sci., 67 (1978) 653-657.
42 I. Vogel, G. Rostock, R. Guetzel and H.D. Faulhaber, Pharmazie, 42 (1987) 165-166.
43 A.J.J. Wood, K. Carr, R.E. Vestal, S. Belcher, G.R. Wilkinson and D.G. Shand, Brit. J. Clin. Pharmacol., 6 (1978) 345-350.
44 J. Chamberlain, Analysis of Drugs in Biological Fluids, CRC Press, Boca Raton, 1987.
45 G.P. Mould, J. Clough, B.A. Morris, G. Stout and V. Marks, Biopharm. Drug Dispos., 2 (1981) 49-57.

Chapter 5.1

GAS-LIQUID CHROMATOGRAPHY OF BETA-BLOCKERS

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5.1.1   INTRODUCTION

   The use of gas chromatography for determination of ß-blockers
in biological samples is founded on the use of sensitive and more
or less selective detectors. In the late sixties, the electron-
capture detector, invented by Lovelock around 1960, had proven to
be extremely useful for measuring halogen-containing trace orga-
nics such as DDT in complex biological or environmental samples.
The first method where a ß-blocker was determined by gas chromato-
graphy with electron-capture detection was published in 1969 [1].
Methods used today [2] are principally the same, although refined
in the design of sample treatment and in instrumentation. Modern
electron-capture detectors are found as reliable work-horses in
continuous use in numerous laboratories. The design of electron
capture detectors has changed little over the past decade,
although research is continuously carried out on the electron-
capture mechanisms [3].

   The combination of gas chromatography with mass spectrometry
dates back to 1957. In 1959, Henneberg [4] used the mass spectro-
meter, focused at a single mass, as a GC detector. The technique
of alternate focusing on selected masses by changing the accele-
rating voltage was applied to GC-MS by Sweeley et al. in 1966 [5].
Ten years later, the technique had found wide acceptance in bio-
medical research, and its use was supported by commercial equip-
ment. Since then, mass spectrometers have continuously developed
and are today powerful, efficient and reliable, but expensive,
instruments.

   The thermoionic ionization detector (TID), which is selective
for nitrogen and phosphorous, is the third type of selective
detector used. It became available in the late seventies. Compared
with its predecessor, the alkali-flame ionization detector (AFID)

with its limited use for quantitative analysis, the TID offered
improved selectivity, signal-to-noise ratio and long-term stabi-
lity. Its sensitivity, for suitable compounds, is between that of
the ECD and the flame ionization detector (FID). The lower sensi-
tivity and the universal response of the FID restricts its use to
samples containing ß-blockers and their metabolites at high con-
centrations, such as urine samples.

5.1.2. DERIVATIZATION OF ß-BLOCKERS FOR GAS CHROMATOGRAPHY

As free bases, ß-blockers are volatile and stable enough to
permit their separation on a gas chromatographic column. This can
be used for toxicological analysis [6] if concentrations are high
and accuracy and precision of quantitative results are not impor-
tant. However, the aminopropanol functionality of the ß-blocker
molecule exhibit adsorptive and reactive [7, 8] properties which
has hindered the separation of underivatized drug in the small
quantities involved in regular bioanalysis. Therefore, derivati-
zation is a main aspect on gas chromatographic analysis of
ß-blockers. Derivatization always includes alteration of the
ß-hydroxyl group, and, except for the case of silylation, also of
the secondary amino group. ß-blockers which exhibit large simi-
larities in their chemical structure can be expected to have simi-
lar, but not identical, properties in derivatization reactions.
The ß-blockers treated in this chapter (Table 5.1.1) are classi-
fied into four groups:
A. ß-blockers having a 1-isopropylamino-3-aryloxy-propan-2-ol
   element and no additional reactive group.
B. ß-blockers containing a 1-tert.butylamino-3-aryloxy-propan-
   2-ol element and no other reactive group.
D. ß-blockers containing additional functional groups which
   might be altered during derivatisation.
C. ß-blockers deviating from the structures A and B which do not
   contain reactive groups other than the secondary amine and
   secondary hydroxyl.

5.1.2.1. Reaction principles

Table 5.1.2 summarizes the different types of derivatisation
reactions used for gas chromatographic separation of ß-blockers.
Chiral reagents are treated in a separate chapter in this book and
are not included here. General information on derivatization is
found in refs. 9-12.
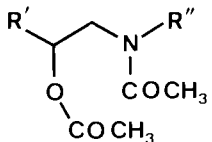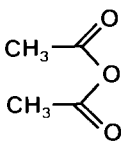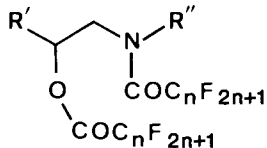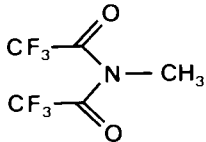
TABLE 5.1.1

Beta-blockers determined by gas chromatography, grouped according to chemical structure. Literature references containing only complementary information on a certain substance are given in brackets.

Substance	References
A. 1-isopropylamino-2-propanols	
Alprenolol	1, 6, (7), 8, 13-19, (20-22, 24)
Acebutolol	25
Befunolol	(24), 26
Betaxolol	8, 27, 28
Bornaprolol	29
Esmolol	30, 31
Exaprolol	32
Flumolol	31
Metoprolol	2, (7), 8, (14), 19, 21, 22, (24), 33-44
Moprolol	45
Oxprenolol	(7), 8, 13, (14), (19-22, 24), 46-49, (50)
Propranolol	(3), 8, (14), 18-20, (21-22, 24, 50), 51-56, 119
Toliprolol	8, (18)
B. 1-tert.-butylamino-2-propanols	
Bufetolol	(20, 24)
Bunitrolol	8, (24), 57
Bunolol	(23)
Bupranolol	8, (20, 24)
Butofilolol	90
Carteolol	8, (20, 24)
Falintolol	58
Penbutolol	8, 59
Tertatolol	60, 61
Timolol	(7), 8, (14, 22, 24), 62-64
Tobanum (trade mark)	65
Xibenolol	66
C. ß-Blockers with additional reactive groups	
Atenolol	(7), 8, (14), 19, (24), 67-71
Desacetylmeti-pranolol	8, 72, 73
EGYT 2427	74
Labetalol	(75, 76)
Nadolol	8, (20), 77-80
Pindolol	(7), 8, 19, 20, (21, 24), 81, 82
Practolol	(7, 14, 18), 50, 83, 84
Sotalol	8, (18)
YM-09538	85
D. Other structures	
Bevantolol	86
Bucindolol	87
Bufuralol	88
CGS 10078B	(89)

TABLE 5.1.2

Derivatization reagents used for GLC determination of beta-blockers. Products are shown for reactions on the aminopropanol moiety of beta-blocker molecules.

Reaction Product	Reagent	References
Acylation		

N,O-bisacetyl derivatives — Acetic anhydride — 8, 56

N,O-bis(perfluoroacyl) derivatives — Trifluoroacetic anhydride (TFAA) — 1, 2, 13, 18, 26, 36, 45, 47-49, 52, 55, 56, 70, 72-74, 83, 84, 119, 24

Pentafluoropropionic anhydride (PFPA) — 29, 31, 34, 54, 69

Heptafluorobutyric anhydride (HFBA) — 13, 19, 27, 34, 38, 39, 49, 51, 58, 67, 68, 86

N-methyl-bis(trifluoroacetamide) (MBTFA) — 37
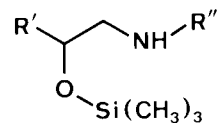
Trifluoroacetylimidazole
(TFAI)   53, 81, 82

CF_3—C(=O)—N (imidazole ring with N)
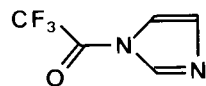
Heptafluorobutyrylimidazole
(HFBI)   63

Silylation

Trimethylsilyl ethers

R'—CH(—O—Si(CH$_3$)$_3$)—CH$_2$—NH—R''

N,O-bis-(trimethylsilyl)acetamide
(BSA)   28, 57

CH_3—C(—O—Si(CH$_3$)$_3$)=N—Si(CH$_3$)$_3$
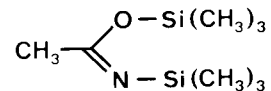
N,O-bis-(trimethylsilyl)-trifluoro-
acetamide (BSTFA)   28, 30, 59, 60, 62

CF_3—C(—O—Si(CH$_3$)$_3$)=N—Si(CH$_3$)$_3$
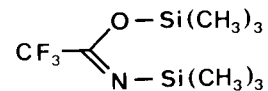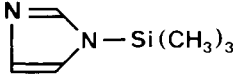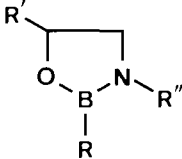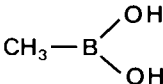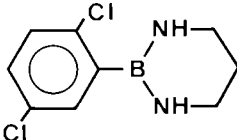
N-methyl,N-trimethylsilyl trifluoro-
acetamide (MSTFA)   64

CF_3—C(=O)—N(CH$_3$)—Si(CH$_3$)$_3$

(TABLE 5.1.2 continued)

126

Reaction Product	Reagent	References
	N-trimethylsilylimidazole (TMSI)	77-79

Mixed silylation/acylation

Reaction Product	Reagent	References
O-trimethylsilyl,N-perfluoroacyl derivatives	BSTFA/TFAA	88
	BSTFA/PFPA	88
	MSTFA/MBTFA	25

Cyclization

Reaction Product		Reagent	References
2-boroxazolidines		methylboronic acid (MBA)	75, 85

	R=CH$_3$		
	R=n-C$_4$H$_7$	n-butylboronic acid (BBA)	20, 75, 76
	R=phenyl	phenylboronic acid (PBA)	20, 76
	R=2,4-dichloro-phenyl	2,4-dichlorobenzeneboronic acid + propanediamine (transboronation reagent)	14

oxazolidinones	phosgene	15, 16, 21, 22, 40, 44

2-(methyl)phosphoxazolidine-2-thiones   sulphur + methyldichlorophosphine   97, 98
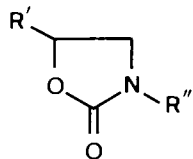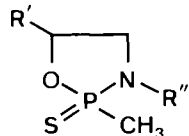
2-(dimethyl)silamorpholines   chloromethyldimethylchlorosilane +   17, 92
1,3-bis(chloromethyl)-1,1,3,3-
tetramethyldisilazane
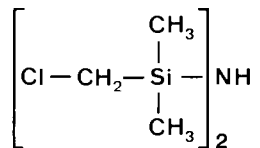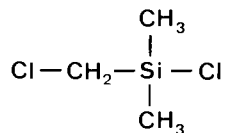
5.1.2.2. Acylation

Acylation implies replacement of an active hydrogen in a molecule by an acyl group, -COR. It is effective on both the hydroxyl and on the amino group of ß-blockers, giving N,O-bis-acyl derivatives. Since derivatives of low volatility should be avoided due to their high elution temperatures, fluorinated acyl derivatives (TFA, PFP, HFB) are generally preferred, although non-halogenated acetyl derivatives have been used.

The reagent can be an acyl halide, an anhydride or a reactive acyl derivative such as amide (MBTFA) or acylated imidazole (TFAI etc.). Generally, the anhydrides are preferred. Acylated imidazoles have been used to avoid the acidic conditions with anhydride reagents, which form the corresponding acid during the reaction. However, traces of water will also produce acid when reacting with an acyl imidazole.

Acylation reactions have many advantageous features: secondary amines are easily converted to amides of high stability; alcohols are converted to esters of moderately high stability. Perfluorinated acylating agents react faster than their non-fluorinated analogues, giving derivatives (and by-products) with higher volatility and high electron-capture detector response. Acetylation, on the other hand, has been used in combination with the nitrogen/phosphorous selective detector. The dominating reactions are those with perfluoroacyl anhydrides.

In many published methods the choice between TFA, PFP and HFB reagents may seem arbitrary, while other papers discuss results from more than one reagent.

Reactivity increases in the order HFBA < PFPA << TFAA. The high reactivity of TFAA has been utilized to derivatize metoprolol by adding ca 2% (v/v) of TFAA directly to the plasma extract [2]. PFPA and HFBA need higher temperature and longer reaction time for complete N,O-bis-acylation. Reaction at the nitrogen is considerably slower for tert.butylaminopropanols than for isopropylaminopropanols, and it is difficult to obtain complete reaction. Nevertheless, xibenolol has been determined in plasma as its N,O-bis-TFA derivative [66] and timolol [63], butofilolol [90] and falintolol [58] have been determined in plasma as their bis-HFB-derivatives. Volatility of the anhydride itself and the corresponding acid is considerably higher for TFAA, which is of practical importance since reagent residues may disturb the chromatographic separation. Problems to remove HFBA by evaporation may make

washing with buffer necessary. Another aspect is availability of
reagents of sufficient purity, which favours TFAA. Reactions may
be performed in the neat reagent but reactivity often is high
enough to ensure complete reaction also in dilute solutions.
Solvents such as ethyl acetate and acetonitrile not only solvate
the reactants but may affect rates and route of reaction. Bases
such as trimethylamine, triethylamine or pyridine may be used as
reaction promotors [91]. Their salts with formed acid have to be
removed after completed reaction by washing with an aqueous buffer
solution. Such a washing step lengthens the time for manual sample
treatment but at the same time removes reagent which otherwise
might have to be removed by evaporation.

5.1.2.3. Silylation

   Trimethylsilylation is, next to perfluoroacylation, the most
common reaction used for GLC of ß-blockers. Other types than tri-
methylsilyl (TMS) have been little used. The O-mono-TMS ethers are
somewhat less volatile than the corresponding N,O-bis-perfluoro-
acyl derivatives. Silyl reagents with larger silyl groups than TMS
will give derivatives with high elution temperatures. Silylation
reagents which introduce electron capturing groups to the
ß-blocker molecule have not found use. In an attemt to use chloro-
dimethylsilyl reagents for this purpose, Hammar [92] found that
with bifunctional compounds, the chloride may react, giving a
non-chlorinated cyclized product (See Table 5.1.2).

   Silyl reagents replace active hydrogens of alcohols, phenols,
carboxylic acids, amines and amides [93]. The ease of reaction, as
well as the hydrolytic stability of the formed derivatives,
decrease in the mentioned order. Steric factors have large
influence on the rate of reaction. Except for the deriatives of
some alcohols, the TMS reagent cannot without risk be removed from
the sample before injection onto the gas chromatographic column.
Silylated amines are themselves active silyl donors. They may be
used in qualitative mass-spectrometric analysis, but they are
difficult to use in quantitative analysis, where a constant, high
yield of the derivative is desired. [94]

   Contrary to acylation, silylation can be used to convert
carboxylic acids into GLC-compatible derivatives and consequently,
acidic metabolites of ß-blockers may be included in the assays.
In quantitative analysis, care should be taken, since the silyl
esters have limited hydrolytic stability and may decompose (chemi-

130

sorb) on the column, as shown by Donike [95]. Successful elution
of silyl esters probably depends on repeated silylation of active
sites in the column by co-injected silyl reagent.

Among the large variety of silyl reagents, only a few have
gained wider use for quantitative ß-blocker determination. The TMS
amides BSA and BSTFA (See Table 5.1.2) combine many of the desired
properties of a reagent: high purity, high silyl donor strength,
fast reactions, high volatility of both reagent and reaction by-
products and compatibility with GLC instruments. BSA and BSTFA
differ little in reactivity. BSTFA and its reaction by-products
containing the trifluoromethyl group are somewhat more volatile
than BSA and its corresponding by-products. This is less critical
when capillary columns are used for separation. BSTFA is claimed
to give less fouling of the flame ionization detector by silica
deposits.

When the aminopropanol side chain is derivatized with a silyla-
ting reagent, only the secondary hydroxyl group reacts quantita-
tively, why silylation of the nitrogen should be avoided. This is
no problem for tert.butylaminopropanols due to steric hindrance.
In fact, most of the applications using TMS derivatives are for
this type of ß-blockers (see Table 5.1.6). Trimethylsilylimidazole
(TMSI) is claimed to be non-reactive against secondary amines and
has been used to avoid undesired N-silylation.

For some ß-blockers containing alcohol functions other than
2-propanol, perfluoroacylation may not give satisfactory results
due to instability of the derivative. This is the case for bufura-
lol [88] where the hydroxyl is vicinal to an aromatic carbon atom.
In this case, a combination of O-trimethylsilylation and
N-perfluoroacylation has been used. Similar instability may be
found for hydroxylated metabolites, such as α-hydroxymetoprolol
[37]. For nadolol, which contains three hydroxyls, trifluoroacety-
lation tends to give a mixture of derivatives of varying composi-
tion, while trimethylsilylation has been used with success
[78-80]. Mixed silylation/acylation has also been used for ace-
butolol, for which treatment with various trifluoroacetylating
reagents alone was unsuccessful [25].

5.1.2.4 Cyclization

Cyclic boronate derivatives of amino-2-propanols, 2-boroxazoli-
dines, have been used in structure elucidation of metabolites with
mass spectrometry [17]. One reason to use cyclization reagents for

quantitation in biological samples would be their claimed selec-
tivity for bifunctional compounds, leaving monofuctional compounds
intact. Cyclic boronate derivatives have good thermal stability
but limited hydrolytic and solvolytic stability. For ß-blockers,
the reagent has to be co-injected. In some cases, the reaction
occurs after injection [14]. Since boronic acids may disturb the
separation and detection processes in GLC, attempts have been made
to choose a reactive derivative. Poole and co-workers [14] used a
propanediamide derivative of 2,4-dichlorobenzeneboronic acid to
produce cyclic derivatives of alprenolol and other ß-blockers with
electron-capturing properties. Detection of the derivatives was
greatly improved compared with the use of the free boronic acid,
but detectability was still limited by the boronic acid formed in
small amount by decomposition of the reagent.

Gyllenhaal et al. introduced phosgene as a cyclization reagent
for GLC and have investigated its properties in conjunction with
ß-blockers and their metabolites [16, 21, 22, 40, 44]. The reac-
tion products, oxazolidine-2-ones, are rather polar, with similar
liquid-liquid distribution properties as the unreacted aminopropa-
nols. The reaction can be carried out either with the sample
matrix present or after isolation of the analyte by solvent
extraction. Quantitative or almost quantitative reaction yields
have been obtained for ß-blockers such as alprenolol and metopro-
lol, while lower yields were observed for hydroxylated metabolites
[40]. Oxazolidinones may be used in conjunction with nitrogen-
selective detection or mass spectrometry. They have also been used
for enantiomer separation on chiral phases [96].

Jacob et al. applied a two-step reaction with methyldichloro-
phosphine and sulphur in presence of triethylamine to form cyclic
derivatives with several ß-blockers. The derivatives, 2-(methyl)-
phosphoxazolidine-2-thiones, had non-polar character and good
chromatographic as well as mass spectral properties [97, 98]. Due
to chirality introduced at the phosphor atom in the reaction, each
ß-blocker produced a pair of diastereomers, which was chromato-
graphically resolved.

5.1.3 ß-BLOCKERS AND THE GAS-CHROMATOGRAPHIC COLUMN

The study of chromatographic behaviour of ß-blockers (after
derivatization) is an important part of GLC assay development, but
systematic investigations within the subject are few.

As a result of chemical derivatization, the functional groups,

which are imcompatible with GLC, are transformed into less active
ones, often sterically shielded by bulky non-polar groups. Acyla-
tion converts the hydroxy and amino groups to ester and amide
groups, respectively. These are still able to interact signifi-
cantly with polar or polarizable groups in the stationary phase or
on the support surface. These interactions diminish in the series
acetyl < TFA < PFP $\leq$ HFB. The TFA derivative of metoprolol is
retained more strongly than the HFB derivative on more polar
methylphenyl stationary phases. On SE-54 (5% phenyl), the reten-
tion times at 200°C are practically identical, and on OV-1 (100%
methyl) they are reversed: TFA elutes before HFB (Table 5.1.3).
This example illustrates that separations can be optimized by
changing either the type of derivative or the stationary phase.
Further, also a change in column temperature may significantly
change selectivity and, in some cases, give reversed elution order
of peaks (Table 5.1.3).

TABLE 5.1.3

Relative retention of the N,O-bis-TFA and N,O-bis-HFB derivatives
of metoprolol on methyl and methylphenyl polysiloxane stationary
phases. Capillary columns were made from fused silica and were
deactivated by high-temperature silylation before coating [43,
104].

Phase	Degree of phenyl substitution	$\alpha = k'_{TFA}/k'_{HFB}$		
		165°C	180°C	200°C
CP-Sil 5	0%	0.83	0.86	0.91
SE-54	5%	0.91	0.94	1.00
SE-54/OV-61	10%	0.97	1.00	1.05
SE-54/OV-61	15%	1.03	1.07	1.12
SE-54/OV-61	20%			1.18
SE-54/OV-61	25%			1.31
OV-61	33%			1.42
DB-17[a]	50%			1.76
OV-17[b]	50%			1.78

[a] column from J & W Scientific, Rancho Cordova, California, USA
[b] column from Quadrex Corporation, New Haven, Connecticut, USA

   Trimethyl-silylation converts the hydroxyl to an ether with
efficient shielding by the bulky, non-polar TMS group, while the
secondary amino group normally remains unaltered. Due to steric
effects, isopropylamino derivatives are somewhat more polar than

tert.butylamino derivatives. For bevantolol which has an ethylene group bonded to the nitrogen, the retention of the TMS derivative on SE-54 at 200°C is roughly double that of the bis-PFP derivative (α = 2.1, ref. 99). The less shielded TMS derivatives also have a tendency to elute with some peak tailing.

Derivatization with phosgene to cyclic oxazolidinones does not introduce non-polar shielding groups, which makes efficient deactivation of columns necessary, especially when using non-polar stationary phases.

Polarity of the derivatives thus influences both retention and peak symmetry. Chemical stability during chromatography, often referred to as "thermal stability", is another important property. Instability is not only due to spontaneous decomposition in the gas phase at elevated temperatures, but more often due to inter-action with molecules or groups in the stationary phase or on the support. Acetyl derivatives of ß-blockers are more stable than perfluoroacyl derivatives. The former have been successfully chromatographed on Carbowax 20M, a polyethyleneglycol phase [56], while already small amounts of Carbowax 20M used for column "deactivation" caused rapid decomposition of perfluoroacyl deriva-tives during elution [41]. TFA derivatives are more sensitive than PFP and HFB derivatives.

The limited chemical stability of N,O-bis-perfluoroacyl deriva-tives makes them incompatible with many stationary phases. With no exception the published methods referred to in Table 5.1.6 employ stationary phases of the polysiloxane type containing methyl, phenyl, vinyl or (one case) trifluoropropyl substituents, the main types being methylpolysiloxane (OV-1, OV-101, SE-30, SP-2100) and methylphenylpolysiloxane (OV-7, OV-17, OV-25). Accordingly the otherwise popular cyano-phases have not been used for perfluoro-acylated beta-blockers.

It has been shown that the instability of N,O-bis-TFA deriva-tives of isopropylaminoalcohols such as metoprolol is not explai-ned by instability of any of the two TFA-acylated groups but due to the proximity of the two groups [41]. Decomposition may occur on columns of the methyl or methylphenyl polysiloxane type, unless the support is prepared by a method which gives proper deactiva-tion. In the author's laboratory, no such deactivation problems were encountered with packed columns, using commercial acid washed and silylated supports of the diatomaceous earth type. However, when open tubular glass columns were introduced, no success was

gained until high-temperature silylation procedures, developed by Grob [100], Welsch [101] and Schomburg [102], were employed [43, 103]. Today high-temperature silylation and other efficient deactivation methods are used by the manufacturers of high-quality capillary columns.

Ahnoff and co-workers reported [41, 103] that the decomposition of perfluoroacyl derivatives during isothermal elution appeared to follow simple first-order kinetics:

$$A = A_0 \times e^{-kt_R'}, \qquad t_{\frac{1}{2}} = \frac{0.693}{k}$$

where A is the area of the eluted peak, A_0 is the area in case of no decomposition, k is a rate constant and t_R' is the corrected retention time (the time spent in the stationary phase). The half-life $t_{\frac{1}{2}}$ is the value of t_R' which corresponds to a loss of 50%. The decomposition rate was independent of the amount injected, and testing with nanogram amounts and flame ionisation detection was preferred. The rate constant k was taken as a measure of the catalytic activity of the column on the solute, as was done by de Nijs et al. [42] for endrine. The rate constant was determined by measuring the area of the eluted peak at two or more different carrier-gas velocities, using a co-injected stable substance as volume marker. Table 5.1.5 contains data from refs. 43, 44 and 103, selected to show typical variations between columns.

For preliminary identification purposes, retention data on ß-blockers recorded in other laboratories may be of interest. Several criteria must be fulfilled, however, in order to obtain reproducible data. The column temperature should be isothermal and accurately measured. Retention should be reported as relative values, with wellknown and readily available reference compounds, as is the case with the Kováts retention index using n-alkanes. If temperature programming has to be used, then the column and instrumental conditions have to be strictly standardized and thoroughly reported. Column length, carrier gas flow rate, phase ratio (between mobile phase and stationary phase) and temperature program rate all affect the elution temperature and therefore affect column selectivity. A recent publication [8] compiles useful mass spectral data for a large number ß-blockers and their urinary metabolites after acetylation. The reported retention indices were recorded using temperature programming at 30°/min and can only serve as a rough guideline for other laboratories. Yamaji [24] reported retention indices for 15 ß-blockers, separated as

their TFA derivatives, as well as underivatized, isothermally on
packed OV-101 and OV-17 columns, but column temperatures were not
given. Poole et al. [14] reported retention indices for 12
ß-blockers and related compounds, separated as cyclic boroxazoli-
dines isothermally on a packed OV-17 column.

TABLE 5.1.4

Decomposition rates (k) and half-lives ($t_{\frac{1}{2}}$) for bis-TFA-metoprolol
measured at 200°C on different 0.3 mm i.d. open tubular columns

Column type	$k \times 10^3 (s^{-1})$	$t_{\frac{1}{2}}$ (min)	Reference
CP-Sil5[a] (100% methyl)	0.27	43	42
OV-1701[a] (6% cyanopropyl, 6% phenyl, 2% vinyl)	6.5	1.8	42
OV-61[b] (33% phenyl)	<0.1	>110	43
OV-61[c] (33% phenyl)	1.3	9	104
	1.1	11	
	1.0	12	
SPB-35[d] (35% phenyl)	1.9	6.1	104
	0.7	17	
DB-17[e] (50% phenyl)	1.2	10	104

[a] Pyrex, HCl-leached, silylated. Static coating with 0.2% solution
[b] Pyrex, NH_3 etched, HCl-leached, silylated. Static coating with 0.2% solution
[c] Fused silica, silylated. Static coating with 0.2% solution.
[d] Fused silica, film thickness 0.25 μm, two columns purchased 1986 from Supelco Inc., Bellefonte, Pennsylvania, USA
[e] Fused silica, film thickness 0.15 μm, purchased 1985 from J & W Scientific, Rancho Cordova, California, USA

5.1.4. DETECTION
5.1.4.1. Electron-capture detection
   The electron-capture detector (ECD), extensively treated in a
volume edited by Zlatkis and Poole [105], has been described as a
chemical reactor detector, the reactants being thermal electrons
and molecules with electron affinity in the gas phase. The concen-
tration of free electrons is measured by using an electrical
field, applied as short pulses in order to avoid collection of
ions. Molecules which capture electrons while passing through the
detector cell decrease the amount of electrons collected during a
pulse, and thus decrease the cell current. In pulse modulated

operation, which is dominating among commercial detectors today, the cell current is kept constant by modulating the pulse frequency. In this case, the detector signal, a current in the picoampere (10^{-12}A) range, is linearly related to the pulse frequency.

Thermal electrons are formed by the impact of ß-particles from a radioactive source, such as Ni-63, on the gas molecules. Nitrogen or a mixture of 5 – 10% methane in argon are used as reactant gas and as carrier gas for packed chromatographic columns. They are less suited as carrier gas for open tubular columns, where radial diffusion rates determine the optimal linear carrier gas velocity, which becomes inconveniently low for nitrogen and argon. Instead helium is preferred for open tubular columns and the reactant gas is added directly to the detector. Due to its detection mechanism, the ECD has been classified as a concentration measuring detector, as opposed to the flame ionisation detector, which measures mass flow. This may be the case with packed columns where the effluent passes undiluted through the detector cell. With open tubular columns, the column effluent (1 – 5 ml/min) is mixed with a constant flow of make-up gas (20 – 60 ml/min), and the detector will function as a mass flow measuring device; higher carrier gas velocity will result in higher detector signal.

The ability of molecules of different chemical structure to collect thermal electrons varies over many orders of magnitude and is the basis for the sensitivity and selectivity of this detector. The electron-capturing properties of N,O-bis-perfluoroacylated amino-2-propanols are determined to some extent by the nature of the perfluoroalkyl groups, with slightly higher signal of the higher homologues: TFA < PFP < HFB. This differs from monoacylated compounds where normally much larger differences are seen between TFA and PFP or HFB. The bis-TFA derivatives of ß-blockers give signals, which are 2 – 3 orders of magnitude higher than for different kinds of mono-TFA derivatives, a property which facilitates their detection in a complex mixture, such as a plasma extract. For PFP and HFB, the differences between the N,O-bis-acyl derivatives and mono-acyl derivatives are smaller, as pointed out by Ervik and co-workers [70, 106]. High electron-capture response after trifluoroacetylation has also been seen for other bifunctional compounds. Clarke et al. [107] noticed the high electron-capture response for N,O-bis-TFA-serine ester, which has the same atom sequence between the TFA groups as ß-blockers. They also observed that the response for bis-trifluoroacetylated diols

increased drastically with a decreasing number of atoms between
the hydroxyl groups. When comparing the ECD response for seven
different trifluoroacetylated ß-blockers, Walle found only minor
differences [18]. Yamaji et al. [24] compared the ECD response for
fifteen ß-blockers with the responses from the thermoionic (TID)
and flame ionisation (FID) detectors. Detection limits with the
ECD were 1 - 2 orders of magnitude lower than with the TID, which
in turn was 1 - 2 orders of magnitude more sensitive than the FID.
Fig. 5.1.1 shows a chromatogram obtained with electron-capture
detection.
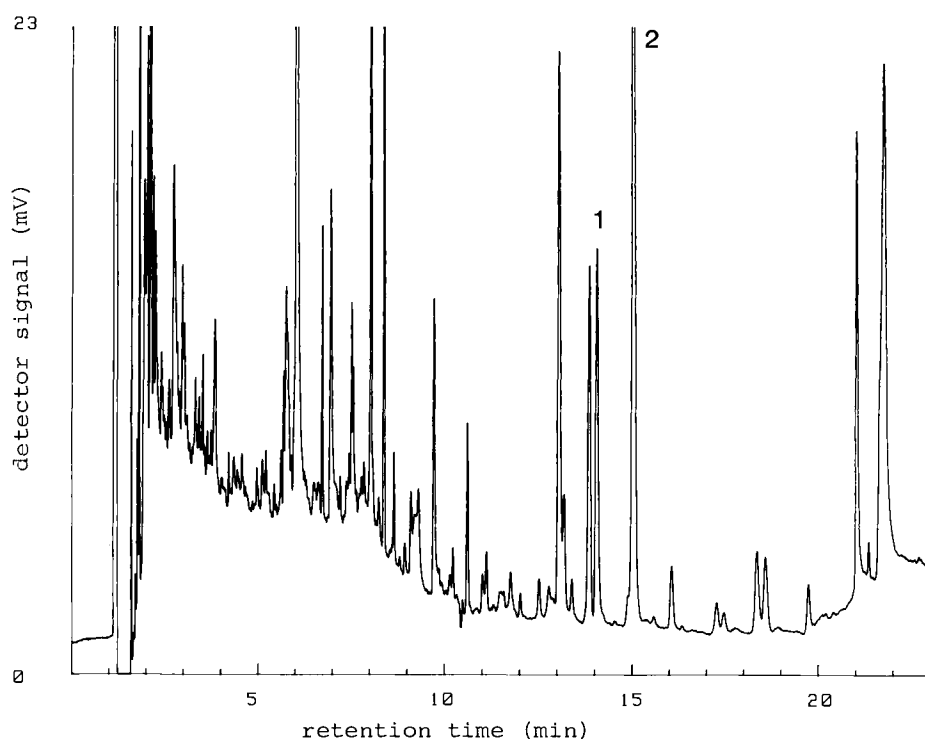
Fig. 5.1.1. An example of the use of high resolution gas
chromatography and electron-capture detection for the determina-
tion of a ß-blocker in plasma after extraction and conversion to
its N,O-bis-pentafluoropropionyl derivative. 1=flumolol,
2=internal standard. Column: 25 m x 0.33 mm i.d. fused silica,
prepared as in Ref. 36, stationary phase 5% phenyl, film thickness
0.17 μm. The inlet pressure was 60 kPa (helium). The initial
column temperature, 100°C, was raised after 1 min at 15°/min to
200°C (10 min isothermal) and further at 30°/min to 250°C (7 min).
The solvent was toluene, and the injected volume, 1.5 μl, corre-
sponded to 3 μl of plasma. Split flow was 20 ml/min (nominal split
ratio 1:10). Flumolol concentration was 33 ng/ml. (Further details
in ref. 31).

5.1.4.2   Selected-ion monitoring

   A mass spectrometer is a device where, at the end of the
process, ions are detected with extremely high sensitivity. In
fact, single ions reaching the secondary electron multiplier are
recorded, and even a low number of ions is sufficient for
achieving statistical significance of the signal. Selectivity is
gained by separating ions according to their mass-to-charge ratio.
Molecules are analysed after ionisation followed by more or less
extensive fragmentation. The ionisation process is, contrary to
the detection, of low efficiency, only a minor portion (10^{-2}-10^{-3})
being ionised. Losses in the mass analyzer are much smaller, but
may be important if the instrument is operated at high mass reso-
lution. Thus, when the mass spectrometer is used as a highly
sensitive gas chromatographic detector, the production of ions at
favourable masses and at high yield is of primary importance.

   It is equally important to minimise detector noise. Noise
caused by ions of other masses reaching the detector is mainly
eliminated by an effective mass analyzer, but it is also essential
to keep the ion source and analyzer at a low level of contamina-
tion. Noise from ions with the same masses as those to be measured
may be due to co-eluted sample components (including earlier
injections) or contaminants from the chromatographic system,
especially decomposition products from the stationary phase.
Electron impact ionsation (EI) is used to produce ions (mainly
positive) at low pressure. Ionisation energies which give high
yield of ions often induce extensive fragmentation, which in turn
decreases selectivity. Chemical ionisation (CI) where a reactant
gas is present in the ionisation chamber, may give higher ion
yields and much less fragmentation. On the other hand, noise often
increases as a result of the higher pressure in the ion source.

   The coupling of the column to the ion-source, the GC-MS inter-
face, has large influence on the performance of the instrument.
The analytes in the column effluent may be incompletely transfer-
red, partially adsorbed or decomposed, peaks may be broadened,
and the interface may be a source of detector noise. Published
ß-blocker assays mostly lack information on what interface was
used. Separators, designed for packed columns, give incomplete
recoveries, typically in the order of 10 - 70%, the rest being
removed together with the carrier gas. Capillary restrictors, used
in open-split interfaces, give a certain flow which, when exceeded
by the column carrier gas, gives incomplete transfer. The inner

surface of a capillary tubing may, if not of an inert material and properly deactivated, cause adsorption or decomposition. For example, glass-lined tubing has caused decomposition of perfluoro-acylated ß-blockers, resulting in changes in mass spectra and drastically reduced signal at the selected masses [108]. For capillary columns, direct coupling to the ion-source gives superior performance, the main drawback being less easy exchange of columns. Comparable results may often be achieved with an optimised open-split interface. Coupling of capillary columns are discussed in ref. 109.

A comprehensive review on quantitative selected ion monitoring of drugs and drug metabolites in biological matrices was made by Garland and Powell in 1982 [120].
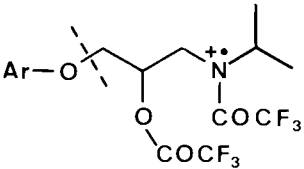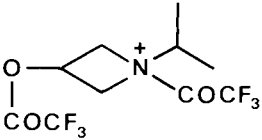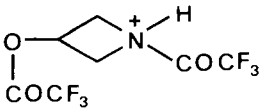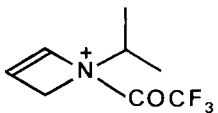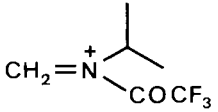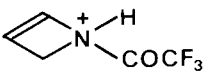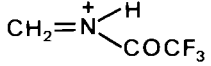
(i) Selected ion monitoring of ß-blockers after acylation. The N,O-bis-perfluoroacyl derivatives of isopropylaminopropanol-type ß-blockers exhibit large similarities in fragmentation pattern, as shown by Garteiz and Walle [50] for alprenolol, oxprenolol, prac-tolol and propranolol. Cleavage of the molecular ion eliminates the aryloxy moiety, the remaining ß-chain giving rise to a number of typical fragments, as shown in Table 5.1.5. For TFA deriva-tives, the masses 308 and 266 are the most abundant, the corre-sponding masses for PFP being 408 and 366 and for HFB 508 and 466. The same fragments are formed from metabolites with intact ß-chain. Fig. 5.1.2 shows selected ion monitoring of metoprolol and two metabolites after trifluoroacetylation. Although most GC-MS assays for the isopropylaminopropanol-type ß-blockers use TFA derivatisation, PFP and HFB derivatives are equally favourable for selected ion monitoring. Chemical ionisation with N_2O as reac-tant gas has been used to determine metoprolol as its N,O-bis-HFB derivative by monitoring m/z 488, which corresponds to a fragment formed by loss of the aryloxy group and of HF from the ß-chain [39]. The main fragment (base peak at m/z 213) was, however, the heptafluorobutyrate ion, which is common for all HFB-derivatives and thus non-selective.

(ii) Selected ion monitoring of ß-blockers after silylation. The O-TMS derivatives of tert.butylaminopropanol and isopropyl-aminopropanol-type ß-blockers give electron impact mass spectra where the fragment at m/z 72 or m/z 86, respectively, often is the base peak and the most favourable for selected ion monitoring, although background noise at these low masses is higher. Other common fragments are M-15 from the loss of CH_3 and M-116 from the

TABLE 5.1.5
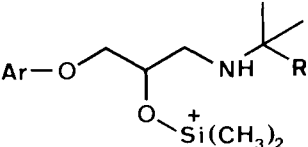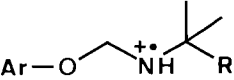
Common positive fragment ions from ß-blockers using electron impact ionisation

A. N,O-bis-acyl derivatives of 1-isopropylamino-3-aryloxy-2-propanols [8, 50, 51]

m/z				Structure
Acetyl	TFA	PFP	HFB	
200	308	408	508	
	266	366	466	
140	194	244	294	
	168	218	268	
98	152	202	252	
72	126	176	226	

(TABLE 5.1.5 continued)

B. O-trimethylsilyl ethers [28]

m/z	Structure
	Ar—O—CH$_2$—CH(O—Si(CH$_3$)$_3$)—CH$_2$—$\overset{+\bullet}{NH}$—C(CH$_3$)$_2$R R = H or CH$_3$
M-15	Ar—O—CH$_2$—CH(O—$\overset{+}{Si}$(CH$_3$)$_2$)—CH$_2$—NH—C(CH$_3$)$_2$R
M-116	Ar—O—CH$_2$—$\overset{+\bullet}{NH}$—C(CH$_3$)$_2$R
101	CH$_2$=CH—O—$\overset{+}{Si}$(CH$_3$)$_2$
72, 86	CH$_2$=$\overset{+}{NH}$—C(CH$_3$)$_2$R

142
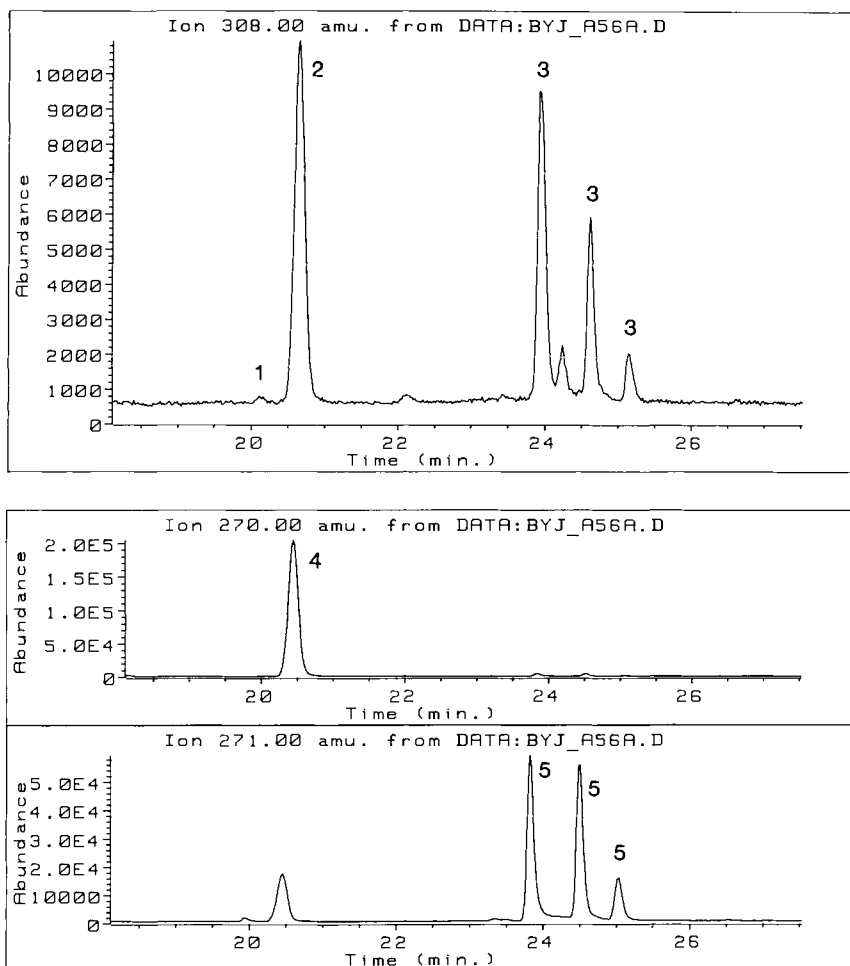
Fig. 5.1.2.   An example of high-resolution gas chromatography and
selected-ion monitoring. Metoprolol and two metabolites were
determined after extraction from an authentic human plasma sample
and subsequent trifluoroacetylation according to ref. 37.
1 = O-demethylmetoprolol (less than 0.3 ng/ml), 2 = metoprolol
(8 ng/ml), 3 = α-hydroxymetoprolol (18 ng/ml), 4 = D_4-metoprolol,
5 = D_5-α-hydroxymetoprolol. Derivatized α-hydroxymetoprolol gives
rise to more than one peak. (See section 5.1.2.3.) The column was
25 m x 0.25 mm i.d. fused silca, HNO_3-leached, deactivated with
hexanemethyldisilazane and coated with 0.15 μm SE-54 (ref. 104).
It was installed with 135 kPa (helium) inlet pressure and via an
open-split interface to an HP 5970 mass-selective detector with
electron-impact ionization at 67 eV. After splitless injection
(2.5 μl, corresponding to 25 μl plasma) the column temperature was
raised from 120°C at 33°/min and the analytes were eluted iso-
thermally at 170°C. (Ref. 118)

loss of TMS-O-CH=CH$_2$. The latter includes rearrangement of the ß-chain as shown by Hermann and co-workers [28]. (See Table 5.1.5).

Chemical ionisation with NH$_3$ as reactant gas has been used to supress fragmentation and allow monitoring of the quasi-molecular ion [MH]$^+$ of betaxolol [28] and tertatolol [60, 61].

5.1.5. CHOOSING COLUMN DIMENSIONS

The electron-capture detector and the mass spectrometer certainly differ widely in construction and properties. The ECD is the less selective of the two and, as far as ß-blockers are concerned, practically non-tunable and requires more efficient chromatographic separation. With GC-MS, selectivity is largely obtained by careful selection of ionsation conditions and of masses to be monitored. For both GC-MS and GC-ECD, performance in terms of accuracy, precision, sensitivity and analysis time is dependent on proper design of the chromatographic system.

The electron-capture detector does not seriously restrict the selection of carrier-gas flow. Thus columns of widely different dimensions, packed as well as open tubular, may be used. An optimal total flow rate, normally 30 ml/min or more, is maintained by addition of make-up gas. The mass spectrometer, on the other hand, has a limited capacity to receive carrier gas while maintaining the intended pressure in the ion source and analyzer. Using electron-impact ionisation, the capacity may vary from below 1 ml/min of helium (at atmospheric pressure) to above 2.5 ml/min. A flow of 1 ml/min of helium restricts the column diameter to 0.2 mm i.d. or smaller if the whole column effluent is to be led into the MS, directly or via an open split interface. A flow of 2.5 ml/min corresponds roughly to 0.32-mm i.d. columns. With chemical ionisation, higher carrier gas flow rates are allowed provided that the ion source and analyzer are separately pumped, and it may be possible to connect wide bore open tubular columns (0.5 mm i.d.), or even packed columns, directly to the ion source. Separators such as the jet separator are used to connect packed columns with flows above 10 ml/min.

When aiming at a certain degree of chromatographic resolution in shortest possible time, capillary columns are superior to packed columns, and higher speed is obtained with columns of smaller inner diameter, which can be made correspondingly shorter. While modern electron-capture cell designs are compatible with

peak widths below 1 s, selected ion monitoring on more than one
single mass has a cycle time in the order of 0.1 to 0.5 s, which
does not permit precise measurement of chromatograhic peaks
shorter than 1 - 5 s.

When high system sensitivity is desired for the determination
of trace amounts in dilute solutions, thin film open tubular
columns are preferred. Column bleeding affects the detector noise
level in both MS and ECD signals and is minimized by using thin
films which means a low mass of stationary phase and low elution
temperatures. If not needed for their higher separation effi-
ciency, very narrow columns are not attractive since smaller
sample volumes are allowed and since elution temperatures are
higher for a given film thickness. For a systematic theoretical
treatment of aspects on optimisation of column dimensions, the
reader should refer to work by Cramers and co-workers [110, 111].

5.1.6 SAMPLE INTRODUCTION

The injection process may be a critical factor affecting
accuracy and precision in quantitative GLC determinations. While
this is true for packed columns for certain classes of compounds,
with badly designed equipment and non-optimal injection condi-
tions, the problem is more general for open tubular columns having
inner diameters of 0.3 mm of less. Most problems arise from two
circumstances: the low volumetric flow to the columns and the
undesired heating of the syringe needle. An excellent summary of
the understanding of processes involved has been made by Schomburg
[112]. A number of injection techniques have been developed to
meet different needs. The classical techniques of split and split-
less injection have been thoroughly penetrated and summarized by
Grob [113], who is also the author of a recent volume treating
on-column injection [114]. Another book, edited by Sandra [115]
also describes recent development of other types of injectors.

While underivatised ß-blockers have been shown to react with
co-injected solvents in the injector [7], no specific injection
problems have been reported in connection with derivatized ß-
blockers. By the use of spiked blank samples for calibration,
systematic errors are largely avoided and the remaining problem is
that of precision. With optimized operation conditions and use of
internal standards, an imprecision in the instrumental analysis of
2 - 3 per cent relative standard deviation (RSD) is realistic for
both split and splitless injection. If higher precision is needed,

cold on-column and temperature programmed injectors could be use-
ful. On-column injection is more easily carried out on wide bore
(0.5 mm) capillary columns, especially if autoinjectors are to be
used. However, the impressive precision achieved for test mixtures
(less than 0.5% RSD) has not been demonstrated for minor compo-
nents in complex samples.

The chromatogram in Fig. 5.1.1 was achieved using split injec-
tion at a nominal split ratio of 1:10. The column was initially
held at a temperature below the boiling point of the solvent,
resulting in an effective split ratio of ca 1:2.5. By temporarily
closing the split during injection (and for 1 min after), a larger
sample can be focused on the column, if needed to increase sensi-
tivity.

5.1.7 COMPARISON OF DIFFERENT GLC METHODS

Table 5.1.6 compiles some key data on published GLC methods for
quantitative determination of ß-blockers in biological samples.

Sample pretreatment consists of more or less elaborate isola-
tion and derivatisation procedures, and instrumental analysis
combines chromatographic separation and more or less selective
detection. Taken together in an analytical method, they determine
what accuracy, precision and sensitivity can be reached. Published
methods can be roughly divided into two categories: those which
use a single extraction step to isolate the ß-blocker from the
bulk of the sample matrix, and those which have extra purification
steps, such as back extraction, to facilitate the subsequent
chromatographic separation and detection.

The high selectivity of selected ion monitoring makes MS
methods less prone to interference from other sample components,
and can be used to simplify sample pretreatment or chromatographic
separation. As seen in Table 5.1.6, however, MS methods use exten-
sive sample pretreatment as frequently as do ECD methods. MS
methods often involve injection of a larger portion of the sample,
corresponding to 10 - 50 µl of plasma, while 1 - 10 µl seems to be
sufficient in most cases for the ECD. The heavier sample load in
GC-MS analyses is probably not always inevitable but may become
necessary if instruments are already in a non-optimal state of
contamination. Careful instrumental optimisation including the gas
chromatographic system can increase sensitivity and decrease the
required sample load, which is beneficial especially when a larger
number of samples is to be analysed.

If methods are grouped according to the type of reagent, one can observe the following pattern: perfluoracylation is common for 1-isopropylamino type compounds, while trimethylsilylation and, as a consequence, MS detection, is common for 1-tert.butylamino type compounds. Quantitative yields are easily obtained from these reactions, as opposed to acylation of 1-tert.butylaminopropanols or silylation of 1-isopropylaminopropanols which may give rise to problems, as discussed in Section 5.1.2. Further, proper elution or O-trimethylsilylated 1-isopropylaminopropanols may be difficult for very small amounts injected (see Section 5.1.3).

Perfluoroacylation allows both ECD and MS detection. Published GC-MS methods mostly use trifluoracetylation, possibly due to lower performance of older mass spectrometers at the higher masses used to monitor PFP- and HFB-derivatives. An estimate of all factors of importance, such as ease of reaction, volatility of the reagent and its by-products, stability of the derivatives in solution and on-column, and selectivity of detection, suggests that PFPA should be considered as an alternative to TFAA and HFBA more often than has been the case.

Some trends of importance for the future analytical work on ß-blockers can be seen. On the one hand the application of high-resolution gas chromatography employing high-quality columns with selective stationary phases is not yet fully realised. On the other hand, the development of mass spectrometric instrumentation is very promising and today more spectacular than the evolution of gas chromatography. Mass selective detectors become easy-to-use work-horses for the routine laboratory. New principles, such as the ion-trap technology [116], are under development, which may lead to advanced instruments of simpler, and potentially cheaper, mechanical construction than conventional mass spectrometers. Sophisticated instrumentation, such as multi-stage mass spectrometers [73, 117] and high-resolution mass spectrometers become realistic alternatives for very demanding analytical problems, or may be employed to further simplify sample pretreatment and chromatographic separation. The gas chromatograph remains, however, the ideal MS inlet for volatile substances, and good chromatography will remain a key to accurate and precise analytical results.

TABLE 5.1.6

Gas chromatographic methods for the determination of beta-blockers in biological samples

Reference	Analyte	Pre-treatment[a]	Reagent[b]	Column type	Detection[c]	Remarks
A. 1-Isopropylamino-2-propanols						
1 (1969)	Alprenolol	E	TFAA	packed, QF-1	ECD	2 ng in 4 ml determined
6 (1978)	Alprenolol	Amberlite XAD-2	none	packed, OV-17/ SilarCP	FID	toxicological application
13 (1979)	Alprenolol Oxprenolol	E+W	HFBA	capillary, OV-101	ECD	2 pg detected
14 (1980)	Alprenolol	E	"transborona-tion reagent"	packed, OV-17	ECD	2.5 ng/ml detected
15 (1985) 16 (1987)	Alprenolol+ metabolites	(E)	$COCl_2$	capillary, CP-Sil 8	TID	derivatisation directly in the sample or after extraction
25 (1976)	Acebutolol + metabolite	E+B+E	1. MSTFA 2. MBTFA	packed, Dexsil 410	ECD	O-TMS, N-TFA
26 (1979)	Befunolol	E+B+W+E	TFAA	packed, OV-25	ECD	2.5 ng/ml detected
27 (1983)	Betaxolol	E+B+W+E	HFBA	capillary, OV-101	ECD	0.5 ng/ml plasma determined
28 (1984)	Betaxolol	E+B+E	BSA or BSTFA	packed, OV-17/ SE-30	MS(CI)	m/z 380 measured 1 ng/ml deter-mined

(TABLE 5.1.6 continued)

Reference	Analyte	Pre-treatment[a]	Reagent[b]	Column type	Detection[c]	Remarks
29 (1980)	Bornaprolol	E+B+E	PFPA	packed, OV-17	ECD	5 ng/ml determined
30 (1984)	Esmolol	E+B+E	BSTFA	packed, SP 2250	MS(EI)	base peak m/z 251 measured
31 (1985)	Esmolol Flumolol	E	PFPA	capillary, CP-Sil 8	ECD	1.5 ng/ml determined
32 (1982)	Exaprolol	E+B+E	TFAA	packed, OV-1	ECD	
33 (1976)	Metoprolol	E	TFAA	packed, JXR	ECD	5 ng/ml determined
2 (1986)	Metoprolol	E	TFAA	capillary, 10% phenyl	ECD	3 ng/ml determined
35 (1980)	Metoprolol metabolites	E	TFAA or HFBI	packed, OV-1 or OV-17	ECD	10 ng/ml determined (plasma and urine)
36 (1981)	Metoprolol	E+B+E	TFAA	packed, OV-17	ECD	3 ng/ml determined
37 (1981)	Metoprolol+ metabolites	E	MBTFA + N-methylimida-zole	packed, OV-101 or OV-17	MS(EI)	0.3 ng/ml determined
38 (1983)	Metoprolol	E+B+E	HFBA+pyridine	packed, OV-1	ECD	10 ng/ml determined
39 (1985)	Metoprolol	E	HFBA+pyridine	packed, OV-1	MS(NICI)	em/z 488 measured

21 (1983)	Metoprolol		$COCl_2$	packed, Hi-EFF- 8BP or capillary, Carbowax 20M	TID	"extractive derivatization"
40 (1984)	Metoprolol+ metabolites		1. $COCl_2$ 2. BSA	packed, Hi-EFF- 8BP or capillary, SE-54	TID/FID	urine samples 1.5 µg/ml (4 µmol/l) detected
45 (1980)	Moprolol	E+B+E	TFAA	packed, SE-30	ECD	
48 (1974) 47 (1976)	Oxprenolol	E+B+E+W	TFAA	packed, OV-101 or JXR	ECD	10 ng/ml determined
46 (1983)	Oxprenolol	E+B+E	HFBA+pyridine	packed, SE-30	ECD	10 ng/ml determined
49 (1984)	Oxprenolol	E	HFBA+pyridine	packed, OV-17	MS(NICI)	m/z 488 measured 6 ng/ml deter- ιined
51 (1973)	Propranolol	E+B+W+E	HFBA	packed, OV-17	ECD	. ng/ml detected
18 (1974)	Propranolol	E+B+E	HFBA+ trimethylamine	packed, OV-17	ECD	0.1 ng/ml detec- ted, 0.5 ng/ml determined
52 (1975)	Propranolol +metabolite	E	TFAA + trimethylamine	packed, OV-1	MS(EI)	m/z 308 measured 50 pg detected 1 ng/ml deter- mined

(TABLE 5.1.6 continued)

150

Reference	Analyte	Pre-treatment[a]	Reagent[b]	Column type	Detection[c]	Remarks
55 (1976)	Propranolol +metabolites	E+B+E	TFAA + trimethylamine	packed, OV-17+ OV-1 (1:2)	ECD	brain tissue 10 ng/g determined
54 (1977)	Propranolol	E+B+E	PFPA+pyridine	packed, OV-1	ECD	
53 (1978)	Propranolol+ metabolites	Amberlite XAD-2	1. TFAI 2. diazomethane	packed, SP-2100	MS(EI) repetitive scans	urine samples
56 (1980)	Propranolol	E	acetic anhydride	capillary, Carbowax 20M	TID	5 ng/ml determined
20 (1982)	Propranolol Pindolol	E	BBA + PBA	packed, OV-17	TID	1.5-4 pg detected 1-2 ng/ml determined
119 (1984)	Propranolol	E	TFAA + trimethylamine	packed, OV-101	ECD	5 ng/ml determined

B. 1-tert.Butylamino-2-propanols

Reference	Analyte	Pre-treatment[a]	Reagent[b]	Column type	Detection[c]	Remarks
57 (1978)	Bunitrolol	E+B+E	BSA + pyridine	packed, OV-17	MS(EI)	m/z 305 measured 160 pg detected 0.8 ng/ml determined
90 (1983)	Butofilolol	E+B+E	HFBA+ trimethylamine	packed, OV-17	ECD	20 ng/ml detected
58 (1987)	Falintolol	E	HFBA	capillary, SE-30	ECD	

59 (1977)	Penbutolol	E	BSTFA + tri-methylchloro-silane	packed, OV-1 with KOH	FID	2 ng detected (15 ng/ml plasma)
60 (1985)	Tertatolol	E+B+W+E	BSTFA	packed, SE-30	MS(CI)	m/z 368 measured 1 ng/ml determined
61 (1987)	Tertatolol+ metabolites	E+B+E	BSTFA	packed, SE-30	MS(CI)	
63 (1975)	Timolol	E+B+W+E	HFBI	packed, OV-17	ECD	2 ng/ml determined
62 (1980)	Timolol	S+E+B+E	BSTFA+pyridine	capillary, SE-30	MS(EI)	m/z 86 measured 0.5 ng/ml detected
64 (1981)	Timolol	E+B+E	MSTFA	capillary, OV-1	MS(EI)	m/z 86 measured
65 (1981)	Tobanum®	E+B+E	TFAA+ trimethylamine	packed, OV-1	ECD	1 ng in 3 ml plasma detected
				packed, OV-101	MS(EI)	m/z 266 measured
66 (1985)	Xibenolol+ metabolites	E+B+E	TFAA	packed, OV-17	MS(EI)	0.5 ng/ml detected

C. ß-Blockers with additional reactive groups

67 (1975)	Atenolol	E+B+W+E	HFBA	packed, UCW 98	ECD	amide converts to nitrile
68 (1975)	Atenolol	W+E+ charcoal	HFBA	packed, UCW 98	ECD	20 ng/ml deter-mined

(TABLE 5.1.6 continued)

Reference	Analyte	Pre-treatment[a]	Reagent	Column type	Detection	Remarks
69 (1978)	Atenolol	E+B+E	PFPA+pyridine	packed, OV-1	ECD	10 ng/ml determined
70 (1980)	Atenolol	E	TFAA	packed, OV-1	ECD	5 ng/ml determined
72 (1982)	Desacetyl-metipranolol	E	TFAA	packed, SE-30	MS(CI)	2 ng/ml determined
73 (1981)	Desacetyl-metipranolol	E	TFAA	capillary	MS-MS(CI)	0.1 ng/ml determined
78 (1978)	Nadolol	E+B+W+freeze drying	TMSI	packed, OV-1	MS(EI)	0,0,0-tris-TMS m/z 86 measured 7 ng/ml detected
79 (1984)	Nadolol	S	1. TMSI+ pyridine 2. TFAA	packed, SP-2100 DB	MS(EI)	m/z 86 measured 0.6 ng/ml determined
77 (1986)	Nadolol	S+XAD-2	TMSI+pyridine	capillary, DB-17	MS(EI)	m/z 86 measured 0.6 ng/ml determined
82 (1980)	Pindolol	E+B+E	TFAI + trimethylamine	packed, OV-17	ECD	N,N,O-tris-TFA 2.5 ng/ml determined
81 (1980)	Pindolol	E+B+E	TFAI + trimethylamine	capillary, OV-1	ECD	1 ng/ml determined

83 (1970) 84 (1975)	Practolol	H+E	TFAA	packed, SE-30	ECD	N,N,O-tris-TFA 10 ng/ml deter- mined
85 (1983)	YM-09538	E	MBA	packed, OV-7	TID	urine samples

D. Other structures

86 (1984)	Bevantolol	W+E	HFBA	packed	ECD	10 ng/ml detected
87 (1986)	Bucindolol+ metabolites	E	MSTFA	capillary, DB-5	MS(EI)	2 ng/ml determined
88 (1976)	Bufuralol+ metabolites	E+B+E	1. BSTFA 2. PFPA	packed, OV-1 + OV-17 (1:1)	ECD	10 ng/ml determined
			1. BSTFA 2. TFAA	packed, OV-17	MS(EI)	m/z 247 measured 1 ng/ml determined

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[a] E = solvent extraction, B = back extraction to water at low pH, W = wash step, S = solid phase extraction, H=hydrolysis

[b] For abbreviations, see Table 5.1.2

[c] TID= thermoionic ionisation detection EI = electron impact ionisation, CI = chemical ionisation, NI = negative ion, MS-MS = tandem mass spectrometry

154

REFERENCES

1   M. Ervik, Acta Pharm. Suecica, 6 (1969) 393.
2   M. Ervik, K. Kylberg-Hanssen and L. Johansson, J. Chromatogr., 381 (1986) 168.
3   E.C.M. Chen, W.E. Wentworth, E.Desai and C.F. Batten, J. Chromatogr., 399 (1987) 121.
4   D. Henneberg, Z. Anal. Chem., 170 (1959) 365.
5   C.C. Sweeley, W.H. Elliot, J. Fries and R. Ryhage, Anal. Chem., 38 (1966) 1549.
6   S.J. Dickson, J.M. Muirhead and P.E. Nelson, J. Anal. Toxicol., 2 (1978) 242.
7   V. Mok, L.V. Bui and L.T. Chan, J. Chromatogr., 393 (1987) 335.
8   H. Maurer and K. Pfleger, J. Chromatogr., 382 (1986) 147.
9   K. Blau and G. King (Eds.), Handbook of derivatives for chromatography. Heyden, London, 1978.
10 S. Ahuja, J. Pharm. Sci., 65 (1976) 163.
11 J.D. Nicholson, Analyst, 103 (1978) 193.
12 C. Poole and S.A. Schuette, Contemporary Practice of Chromatography, Elsevier, Amsterdam, 1984.
13 D. DeBruyne, H. Kinsun M.A. Moulin and M.C. Bigot, J. Pharm. Sci., 68 (1979) 511.
14 C.F. Poole, L. Johansson and J. Vessman, J. Chromatogr., 194 (1980) 365.
15 O. Gyllenhaal, J. Chromatogr., 349 (1985) 447.
16 O. Gyllenhaal, J. Chromatogr., 413 (1987) 270.
17 K.-J. Hoffmann, A. Arfwidsson, K.O. Borg and I. Skånberg, Biomed. Mass Spectrom., 5 (1978) 634.
18 T. Walle, J. Pharm. Sci., 63 (1974) 1885.
19 F. Susanto and H. Reinauer, Fresenius Z. Anal. Chem., 318 (1984) 425.
20 T. Yamaguchi, Y. Morimoto, Y. Sekine and M. Hashimoto, J. Chromatogr., 239 (1982) 609.
21 O. Gyllenhaal and J. Vessman, J. Chromatogr., 273 (1983) 129.
22 O. Gyllenhaal and J. Vessman, J. Chromatogr., 395 (1987) 445.
23 F.-J. Leinweber, L.J. Haynes, M.C. Crew and F.J. Di Carlo, J. Pharm. Sci., 60 (1971) 1512.
24 A. Yamaji, K. Kataoka, N. Kanamori, M. Oishi and E. Hiraoka, Yakugaku Zasshi, 105 (1985) 1179.
25 P.J. Meffin, S.R. Harapat and D.C. Harrison, Res. Commun. Chem. Pathol. Pharmacol., 15 (1976) 31.
26 K. Kawahara and T. Ofuji, J. Chromatogr., 168 (1979) 266.
27 J. Ganansia, G. Gillet, P. Padovani and G. Bianchetti, J. Chromatogr., 275 (1983) 183.
28 Ph. Hermann, J. Fraisse, J. Allen, P.L. Morselli and J. P. Theuot, Biomed. Mass Spectrom., 11 (1984) 29.
29 N. Bernard, G. Cuisinaud, C. Jozefczak, M. Seccia, N. Ferry and J. Sassard, J. Chromatogr., 183 (1980) 99.

30 C.Y. Sum and A. Yacobi, J. Pharm. Sci., 73 (1984) 1177.
31 G. Holm, K. Kylberg-Hanssen and L. Svensson, Clin. Chem., 31 (1985) 868.
32 A. Schultzová, O. Gattnar, D. Kováčová and Z. Mahrla, Ceskoslov. Farm., 31 (1982) 64.
33 M. Ervik, Acta Pharmacol. Toxicol., 36 (Suppl. V) (1975) 136.
34 S. Zak, F. Honc and T.G. Gilleran, Anal. Lett., 13 (1980) 1359.
35 C.P. Quarterman, M.J. Kendall and D.B. Jack, J. Chromatogr., 183 (1980) 92.
36 C.D. Kinney, J. Chromatogr., 225 (1981) 213.
37 M. Ervik, K.-J. Hoffmann and K. Kylberg-Hanssen, Biomed. Mass Spectrom., 8 (1981) 322.
38 A. Sioufi, F. Leroux and N. Sandrenan, J. Chromatogr., 272 (1983) 103.
39 D. Gaudry, D. Wantiez, J. Richard and J.P. Metayer, J. Chromatogr., 339 (1985) 404.
40 O. Gyllenhaal and K.-J. Hoffman, J. Chromatogr., 309 (1984) 317.
41 M. Ahnoff, M. Ervik and L. Johansson, in R.E. Kaiser (Ed.), Proc. 4th Int. Symp. Capillary Chromatography, Hindelang, May 3-7, 1981, Hüthig, Heidelberg, 1981, pp. 487-504, 903.
42 R.C.M. de Nijs, J.J. Franken, R.P.M. Dooper, J.A. Rijks, H.J.J.M. deRuwe and F.L. Schulting, J. Chromatogr., 167 (1978) 231.
43 M. Ahnoff and L. Johansson, Chromatographia, 19 (1984) 151.
44 K.-J. Hoffman, O. Gyllenhaal and J. Vessman, Biomed. Environ. Mass Spectrom., 14 (1987) 543.
45 J.P. Desager, J. High Resolut. Chromatogr. Chromatogr, Commun., 3 (1980) 129.
46 A. Sioufi, D. Colussi and P. Mangoni, J. Chromatogr., 278 (1983) 185.
47 P.H. Degen and W. Reiss, J. Chromatogr., 121 (1976) 72.
48 D.B. Jack and W. Riess, J. Chromatogr., 88 (1974) 173.
49 J. Godbillon, A. Gerardin, D. Gaudry, J.P. Metayer, J. Richard and D. Wantiez, in J.M. Aiache and J. Hirtz (Eds.), Proc. 2nd Eur. Congr. Biopharm. Pharmacokinet. 1984, Salamanca, April 24-27, 1984, L'Université, Clermont-Ferrand, 1984.
50 D.A. Garteiz and T. Walle, J. Pharm. Sci., 61 (1972) 1728.
51 E. Di Salle, K.M. Baker, S.R. Bareggi, W.D. Watkins, C.A. Chidsey, A.Frigerio and P.L. Morselli, J. Chromatogr., 84 (1973) 347.
52 T. Walle, J. Morrison, K. Walle and E. Conradi, J. Chromatogr., 114 (1975) 351.
53 V.T. Vu and F.P. Abramson, Biomed. Mass Spectrom., 5 (1978) 686.
54 R.E. Kates and C.L. Jones, J. Pharm. Sci., 66 (1977) 1490.
55 D.A. Saelens, T. Walle, P.J. Privitera, J. Chromatogr., 123 (1976) 185.
56 A.G. De Boer, D.D. Breimer and J.M. Gubbens-Stibbe, Pharm. Weekblad Sci. Edit., 2 (1980) 101
57 H.J. Förster and V. Häselbarth, in A.P. de Leenheer, R.R. Roncucci and C. van Peteghem (Eds.), Quantitative Mass Spectrometry in Life Science II. Proc. Second. Int. Symp., State Univ. of Ghent, June 13-16, 1978. Elsevier, Amsterdam, 1978, pp. 275-285.
58 J. Himber, G. Andermann, M. Bouzoubaa and G. Leclerc, J. Chromatogr. Sci., 25 (1987) 33.
59 J.F. Guidicelli, C. Richer, M. Chauvin, N. Idrissi and A. Berdeaux, Brit. J. Clin. Pharmacol., 4 (1977) 135.

156

60 S. Staveris, P. Blaise, C. Efthymiopoulos, M. Schneider, G. Jamet, L. Jung and J.C. Koffel, 339 (1985) 97.
61 C. Efthymiopoulos, S. Staveris, F. Weber, J.C. Koffel and L. Jung, J. Chromatogr., 421 (1987) 360.
62 J.R. Carlin, R.W. Walker, R.O. Davies, R.T. Ferguson and W.J.A. VandenHeuvel, J. Pharm. Sci., 69 (1980) 1111.
63 D.J. Tocco, F.A. deLuna and A.E.W. Duncan, J. Pharm. Sci., 64 (1975) 1879.
64 J.B. Fourtillan, M.A. Lefebre, J.Girault and Ph. Courtois, J. Pharm. Sci., 70 (1981) 573.
65 E. Tomori and I. Elekes, J. Chromatogr., 204 (1981) 355-360.
66 S. Honma, T. Ito and A. Kambegawa, Chem. Pharm. Bull., 33 (1985) 760.
67 B. Scales and P.B. Copsey, J. Pharm. Sci., 27 (1975) 430.
68 J.O. Malbica and K.R. Monson, J. Pharm. Sci., 64 (1975) 1992.
69 S.H. Wan, R.F. Maronde and S.B. Matin, J. Pharm. Sci., 67 (1978) 1340.
70 M. Ervik, K. Kylberg-Hanssen and P.-O. Lagerström, J. Chromatogr., 182 (1980) 341.
71 Y. Matsuki, T. Ito, S. Komatsu and T. Nambara, Chem. Pharm. Bull., 30 (1982) 196.
72 R. Endele, M. Senn and U. Abshagen, J. Chromatogr., 227 (1982) 187.
73 J.R.B. Slayback, R. Schubert, U. Abshagen, R. Endele, M. Senn, Finnigan Topic 8161, Finnigan MAT, 1981.
74 K. Lakszner, L. Szepesy and L. Vida, Chromatographia, 19 (1984) 304.
75 T.J. Cholerton, J.H. Hunt and M. Martin-Smith, J. Chromatogr., 333 (1985) 178.
76 G. Munro, J.H. Hunt, L.R. Rowe, M.B. Evans, Chromatographia, 11 (1978) 440.
77 M. Ribick, E. Ivashkiv, M. Jemal and A.I. Cohen, J. Chromatogr., 381 (1986) 419.
78 P.T. Funke, M.F. Malley, E. Ivashkiv and A.I. Cohen, J. Pharm. Sci., 67 (1978) 653.
79 A.I. Cohen, R.G. Devlin, E. Ivashkiv, P.T. Funke and T.
80 A.I. Cohen, M. Jemal, E. Ivashkiv and M. Ribick, J. Chromatogr., 416 (1987) 445.
81 M. Guerret, J. Chromatogr., 221 (1980) 387.
82 M. Guerret, D. Lavene and J.R. Kiechel, J. Pharm. Sci., 69 (1980) 1191.
83 B. Scales and M.B. Cosgrove, J. Pharm. Exper. Ther., 175 (1970) 338.
84 J.P. Desager, C. Harvengt, J. Pharm. Pharmacol., 27 (1975) 52.
85 H. Kamimura, H. Sasaki and S. Kawamura, J. Chromatogr., 275 (1983) 81.
86 E.J. Randimitis, C. Nelson and A.W. Kinkel, J. Chromatogr., 308 (1984) 345.
87 M.J. Bartek, E.H. Kerns, R.E. Gammans and D.G Gallo, J. Chromatogr., 377 (1986) 183.
88 R.J. Francis, P.B. East, S.J. McLaren and J. Larman, Biomed. Mass Spectrom., 3 (1976) 281.
89 F.R. Goodman, G.B. Weiss, M.E. Hurley and V.M. Traina, in A. Scribine (Ed.), New Drugs Annual: Cardiovascular Drugs, Vol. 3, Raven Press, New York, 1985, pp. 121-131.
90 J.Ph. Jeanniot, G. Houin, P. Ledudal, D. Berthet, D Lusseau, P. Gros and J.P. Tillement, J. Chromatogr., 278 (1983) 301.
91 T. Walle and H. Ehrsson, Acta Pharm. Suecica, 7 (1970) 389.
92 C.-G. Hammar, Biomed. Mass Spectrom., 5 (1978) 25.

93   A.E. Pierce, Silylation of Organic Compounds, Pierce Chemical Company, Rockford, Illinois, 1968.
94   F. Artigas, E. Martinez and E. Gelpi, J. Chromatogr. Sci., 20 (1982) 75.
95   M. Donike, Chromatographia, 6 (1973) 190.
     McCormick, J. Pharm. Sci., 73 (1984) 1571.
96   W.A. König, K. Ernst and J. Vessman, J. Chromatogr., 294 (1984) 423.
97   K. Jacob, W. Voty, C. Krauss, G. Schnabl and M. Knedel, Biomed. Mass Spectrom., 10 (1983) 175.
98   K. Jacob, G. Schnabl and W. Vogt, Chromatographia, 19 (1984) 216.
99   G. Edvardsson, personal communication.
100 K. Grob, G. Grob and K. Grob Jr., J. High Resolut. Chromatogr. Chromatogr. Commun., 2 (1979) 31.
101 Th. Welsch, W. Engewald and Ch. Klancke, Chromatographia, 10 (1977) 22.
102 G. Schomburg, H. Husmann and H. Borowitzky, Chromatographia, 12 (1979) 651.
103 M. Ahnoff and L. Johansson, J. Chromatogr., 279 (1983) 75.
104 L. Johansson, personal communication.
105 A. Zlatkis and C.F. Poole (Eds.), Electron Capture, Theory and Practice in Chromatography, Elsevier, Amsterdam, 1981.
106 M. Ervik, T. Walle and H. Ehrsson, Acta Pharm. Suecica, 7 (1970) 625.
107 D.D. Clarke, S. Wilk and S.E. Gitlow, in H.A. Szymanski (Ed.), Biochemical Applications of Gas Chromatography, Vol 2, Plenum Press, 1968, pp. 137-163.
108 M. Ervik, personal communication.
109 M.L. Lee, F.J. Yang and K.D. Bartle, Open Tubular Column Gas Chromatography, John Wiley & Sons, New York, 1984.
110 Th. Noy, J. Curvers and C. Cramers, J. High Resolut. Chromatogr. Chromatogr. Commun., 9 (1986) 752.
111 Th. Noy and C. Cramers, in P. Sandra (Ed.), Proc. 8th Int. Symp. Capillary Chromatography, Riva del Garda, May 19-21, 1987, Hüthig, Heidelberg, 1987, pp. 95-109.
112 G. Schomburg, U. Häusig and H. Husmann, J. High Resolut. Chromatogr. Chromatogr. Commun., 8 (1985) 566.
113 K. Grob Jr., Classical Split and Splitless Injection in Capillary Gas Chromatography, Hüthig, Heidelberg, 1986.
114 K. Grob, Jr., On-Column Injection in Capillary Gas Chromatography, Hüthig, Heidelberg, 1987.
115 P. Sandra (Ed.), Sample Introduction in Capillary Gas Chromatography, Hüthig, Heidelberg, 1985.
116 P.H. Dawson, Mass Spectrom. Rev., 5 (1986) 1.
117 R.A. Yost and D.D. Fetterolf, Mass Spectrom. Rev., 2 (1983) 1.
118 B. Yhlen, personal communication.
119 W.-R. Stenzel, G. Michael and L. Lyhs, Pharmazie, 40 (1985) 360.
120 W.A. Garland and M.L. Powell, J. Chromatogr. Sci., 19 (1981) 392.

Chapter 5.2

HPLC DETERMINATION OF BETA-ADRENERGIC BLOCKERS IN BIOLOGICAL FLUIDS

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5.2.1 INTRODUCTION

    The need for sensitive and specific HPLC methods for determination of plasma beta-blocker concentration has grown in recent years as the number of commercially available beta-blocking agents has increased. Unlike gas chromatographic methods which may require derivitization, high-performance liquid chromatography with fluorescence, ultraviolet, or electrochemical detection provides the analytical method of choice for beta-blocking agents. The specificity and sensitivity reflected in low limits of detection with relatively simple sample work-up make HPLC methods suitable for single-dose pharmacokinetic studies, as well as therapeutic drug monitoring during clinical use.

    This chapter reviews HPLC analytical methods for a number of beta-blocking agents, including those in clinical use as well as investigational compounds. The text discusses methodologies available for individual drugs. A summary can be found in the Appendix.

5.2.2   METHODS FOR QUANTITATION OF INDIVIDUAL BETA BLOCKERS

5.2.2.1 Acebutolol

    The reported methods of HPLC analysis for acebutolol[1-6] have all utilized reverse phase chromatography at room temperature with C_{18} packed columns. Both fluorescence[2,3,5] and UV[1,4,6] detection have been used. All reported methods used internal standards for quantification of plasma concentrations, with single step extraction of alkalinized plasma followed by evaporation of the organic phase.[2,6] Fluorescence detection[2] provided a lower limit of sensitivity of 10 ng/ml vs 50 ng/ml for UV detection.[6] Of the three procedures[1,3,4] that involved back-extraction of the organic phase to

sulfuric acid, two utilized UV detection (240 nm;[1] 243 nm.[4]) and one used fluorescence.[3] All of these three methods were designed to enable the measurement of the major metabolites of acebutolol, including diacetolol.

Another method utilizing fluorescence detection[5] required a 2-hour derivitation step with S-(-)-N-trifluoroacetylprolyl chloride. This method was designed to detect the different stereoisomers of acebutolol and diacetolol. The lower limit of detection is well above that of less complex and time consuming methods (50 ng/ml vs 5-10 ng/ml) making this method less desirable for routine plasma level monitoring.

5.2.2.2 Adimolol

A sensitive reverse phase assay performed at room temperature has been reported for adimolol.[7] With back-extraction into sulfuric acid and fluorescence detection, the method is sufficiently sensitive to quantify as little as 0.5 ng/ml of adimolol.

5.2.2.3 Alprenolol

Alprenolol plasma concentrations have been determined by a simple and sensitive reverse-phase assay.[8] The mobile phase of acetonitrile and water contained 1% triethylamine as an amine modifer to improve peak symmetry by blocking silanol groups on the stationary phase. Fluorescence detection was used following back-extraction of the samples into phosphate buffer (pH=1.5). The lower limit of detection was reported as 0.33 ng/ml.

5.2.2.4 Atenolol

Atenolol plasma concentrations have been measured by a variety of methods utilizing UV and fluorescence detection, C_{18}, cyano and silica packed columns, and solid phase and liquid phase extractions.[3,9-14]

Using UV detection at 224 nm, and a cyano column (6 micron) at room temperature, Verghese and Associates[11] developed a rapid extraction procedure using disposable solid phase, cyano extraction columns. The method used practolol as an internal standard and provided a lower limit of detection of 10 ng/ml of plasma. Triethylamine was added to the mobile phase in order to sharpen the peaks on the chromatogram.

Another reported method developed with a cyano column used solid phase extraction columns for plasma extraction, and fluorescence detection.[12] This method has the important drawback of lacking an internal standard.

Sensitive methods performed with C_{18} columns and fluorescence detection have been reported.[3,9,10] Three of these methods require back-extraction into sulfuric acid.[3,9,10] Metoprolol[9] and procainamide[3] were used as internal standards, while another method[10] used no internal standard.

A method requiring only a single extraction step and chromatography on a C_{18} column with fluorescence detection has also been reported.[14] This method provides a lower limit of detection of 5 ng/ml.

Buhring and Garbe[13] reported a procedure using a normal-phase silica column and a reverse phase solvent system of acetonitrile/water/phosphate buffer with fluorescence detection. This method required a more extensive workup than the others, with an extraction step followed by an evaporation step and reconstitution with a wash step and a final evaporation. It provided a lower limit of detection of 5 ng/ml of plasma.

5.2.2.5 Betaxolol

Although plasma concentrations of betaxolol have been determined with several different types of packed columns and temperatures,[15-18] all reported detection methods were fluorescence.

A single extraction step followed by separation on a cyano column at room temperature with fluorescence detection[15] provided a lower limit of detection of 1 ng/ml. This method described a simple routine for automated high-performance liquid chromatography systems.

A reverse phase method[16] using a C_{18} column and fluorescence detection provided a lower limit of detection of 4 ng/ml but required three extraction steps and a final evaporation.

Another method using a C_{18} packed column and fluorescence detection[17] also has been reported. This method involves chromatography at $36°C$ and a series of steps to derivatize betoxolol in order to distinguish the different enantiomers of betaxolol. This method allows quantification of betaxolol down to 0.5 ng/ml of plasma.

A normal phase chromatographic method has also been reported.[18] Using a silica packed column at room temperature and fluorescence detection following a single extraction step, the method produced a lower limit of detection of 5 ng/ml.

5.2.2.6 Bevantolol

An assay method for bevantolol and its hydroxy metabolite in plasma has recently been reported.[19]

This method utilizes a cyano packed column with ultraviolet detection. Extraction of alkalinized plasma was followed by back-extraction into hydrochloric acid. The lower limit of detection was reported to be 26 ng/ml of plasma.

5.2.2.7 Bisoprolol

Bisoprolol levels have been measured in plasma with fluorescence detection following chromatographic separation on a silica packed column with

a reverse phase solvent system of acetonitrile/water/phosphate buffer.[13] This method provided a lower limit of detection of 1-2 ng/ml of plasma.

5.2.2.8 Bopindolol

Bopindolol concentrations in plasma have been determined by measurement of the active hydrolyzed metabolite of bopindolol. A method using fluorescence detection and chromatographic separation on a cyano column has been reported.[20] This method involves the measurement of the active and highly fluorescent metabolite in plasma samples before and after enzyme hydrolysis. Parent drug is then calculated from the difference in these concentrations. This method does not employ the use of an internal standard.

Electrochemical detection was utilized in another reported method.[21] Chromatography was on a 3-micron C_{18} packed column maintained at 40°C. Extraction from plasma was accomplished by solid phase extraction on disposable, C_{18} packed extraction cartridges. This method also only measures the active hydrolyzed metabolite. The electrochemical detection allows for quantification of as little as 0.05 ng/ml of plasma.

5.2.2.9 Bufuralol

Plasma concentrations of bufuralol have been measured using a reverse phase chromatographic system which utilized a C_{18} packed column and fluorescence detection.[22] Three extraction steps with an intermediate wash step were required to provide sufficient sensitivity to quantitate 1 ng/ml using a 0.5 ml plasma specimen.

5.2.2.10 Bunitrolol

Bunitrolol plasma concentrations have been measured in plasma using reverse-phase chromatography on a C_{18} packed column at 40°C with fluorescence detection.[23] The extraction procedure involved alkalinization with phosphate buffer followed by double extractions with diethyl ether and back-extraction into dilute hydrochloric acid. The lower limit of detection was 2.5 ng/ml of plasma.

5.2.2.11 Bupranolol

Ultraviolet detection was used in a reported method for the determination of bupranolol in plasma.[24] Chromatography was carried out on a cyano packed column. A single extraction step with subsequent evaporation and reconstitution in mobile phase proved to be sufficient for providing a lower limit of detection of 1 ng/ml of plasma.

5.2.2.12 Butofilolol

Butofilolol plasma concentrations have been measured by a reverse phase chromatographic system with a C_{18} packed column and ultraviolet detection.[25] The lower limit of detection was estimated to be 20 ng/ml of plasma.

5.2.2.13 Carvedilol

A sensitive assay method for carvedilol in plasma has recently been reported.[26] Separation takes place on a C_{18} packed column at room temperature with a simple mobile phase of dilute phosphoric acid and methanol. Fluorescence detection is used, following back-extraction into sulfuric acid, and the procedure provides sufficient sensitivity to measure as little as 0.4 ng/ml of plasma.

5.2.2.14 Celiprolol

Reverse phase chromatography with C_{18} packed columns has been utilized with both ultraviolet and fluorescence detection.[27] Both methods require back-extraction steps. Fluorescence detection provides a lower limit of detection of 5 ng/ml as compared to ultraviolet detection with a lower limit of 10 ng/ml.

5.2.2.15 Esmolol

Plasma concentrations of esmolol have been determined by a reverse phase chromatographic method with a C_{18} packed column and ultraviolet detection following a single extraction step.[28] A lower limit of 50 ng/ml was estimated, but this method does not use an internal standard.

5.2.2.16 Labetalol

Labetalol plasma concentrations have been measured by simple reverse phase chromatographic methods with C_{18} packed columns and ultraviolet detection.[29,33] A single extraction method[29] provided a lower limit of detection of 40 ng/ml, while a more recent report utilizing back extraction into dilute hydrochloric acid produced a 10 ng/ml lower limit.[33]

Two other methods were reported using a C_{18} packed column but fluorescence detection.[30,31] To maximize fluorescence, one group utilized post-column alkalinization with borate buffer.[30] This requires the use of an additional pump. The method achieves a lower limit of detection of 1 ng/ml of plasma and requires only one extraction step for sample clean up. The other method used two extractions from alkaline plasma and chromatographic separation at 30°C to give a detection limit of 8 ng/ml.[31]

An assay method using a column packed with spherical, macroporous poly(styrene-divinyl benzene)(PRP-1)[32] and fluorescence detection has also been reported. This column allows the use of mobile phases at pH extremes.

The pH of the mobile phase for this method was chosen as 9.5 in order to optimize fluorescence of the labetalol. With a back-extraction step for additional clean-up, the lower limit of detection is 4 ng/ml of plasma.

5.2.2.17 Levobunolol

An assay method for the determination of blood levels of levobunolol has been reported.[34] This method utilizes reverse-phase chromatography on a C_{18} packed column and fluorescence detection. Heptane sulphonic acid was added to the mobile phase to increase peak symmetry. Acetonitrile was added to the fresh blood samples prior to freezing to halt enzymatic conversion of levobunolol to its dihydro metabolite. When defrosted, samples were alkalinized, twice extracted with benzene, and then evaporated. The lower limit of detection was 0.5-1 ng/ml of blood.

5.2.2.18 Medroxalol

Medroxalol plasma concentrations have been measured by ambient temperature reverse phase chromatography on a C_{18} packed column with fluorescence detection.[35] On-line column switching methods requiring two pumps were used for sample cleanup. The lower limit of detection was 50 ng/ml of plasma.

5.2.2.19 Mepindolol

Electrochemical detection methods were used in a reported assay for the determination of plasma concentrations of mepindolol.[36] Sodium dodecylsulfonate was added to the mobile phase for ion-pair formation. Sample workup requires back-extraction into acetic acid followed by two wash steps to provide a lower limit of detection of 1 ng/ml of plasma.

5.2.2.20 Metoprolol

Although metoprolol plasma concentrations have primarily been determined with fluorescence detection, one method has been reported which utilizes ultraviolet detection.[3] This method uses a C_{18} packed column for separation and a back-extraction step for sample cleanup.

C_{18} packed columns were used with fluorescence detection in a number of other reported assay methods.[10,12,39,40,42,43,44] A back-extraction step was used prior to chromatography in two methods[10,44] and produced lower limits of detection of 2 ng/ml[10] and 5 ng/ml[44] although one method did not use an internal standard.[10] Dimethyloctylamine was used[44] as an amine modifier to sharpen the chromatographic peaks.

A single step extraction followed by evaporation was reported in one method[39] and produced a lower limit of detection of 5 ng/ml. Another method reported the same limit with plasma protein precipition followed by a single

extraction.[42] A method for determining metoprolol enantiomers in plasma[43] required a single extraction step that also accomplished the derivitization of the enantiomers with a chiral agent. This method gave a lower limit of detection of 2 ng/ml of plasma. Solid phase extraction with disposable C_{18} packed extraction cartridges was accomplished in one method[12] with a reported limit of detection of 2 ng/ml, although no internal standard was used in this method.

 C_8 stationary phases have been used for chromatographic separation with fluorescence detection.[37,41] One method[37] required chromatography at 40°C in a mobile phase containing l-heptanesulfonic acid as an ion-pairing reagent. With one extraction step, this method provided a lower limit of detection of 5 ng/ml of plasma. Another method[41] involved chromatography at 50°C with a sample workup designed for quantitation of metabolites as well as intact metoprolol, so that the lower limit of detection is a reported 10 ng/ml of plasma.

 Silica packed columns were used in the chromatographic methods reported for two assays.[13,38] Both utilized fluorescence detection. One method[38] used a standard normal phase set-up with a predominantly hexane mobile phase.
 Sample cleanup was accomplished by an organic wash step followed by an extraction step to produce a lower limit of detection of 3 ng/ml of plasma. A second method[13] used a reverse phase solvent system of acetonitrile/water/phosphate buffer. This method requires extensive sample manipulation for cleanup but provides a lower limit of detection of 1-2 ng/ml of plasma.

5.2.2.21 Nadolol

 Reverse phase chromatography assays using both fluorescence[45,46] and ultraviolet detection[47] have been reported. Separation on a poly(styrene-divinyl benzene)(PRP-1) column after solid phase extraction using disposable C_{18} packed extraction cartridges[45] provided a lower limit of detection of 10 ng/ml. Another method with fluorescence detection[46] following a single extraction step and separation on a C_{18} packed column provided a limit of detection of 1 ng/ml of plasma. This method used diethylamine as an amine modifier to sharpen chromatographic peaks.

 A method using ultraviolet detection after a single extraction step followed by separation on a C_{18} packed column produced a lower limit of detection of 20 ng/ml.[47] This assay method successfully employs recirculating eluent flow to minimize the amount of mobile phase needed for chromatography.

5.2.2.22 Oxprenolol

Ultraviolet detection has been used as the means of detection of plasma oxprenolol concentrations in all assay methods reported.[3,48,49] The methods use C_{18} packed columns at room temperature[3,49] or 30°C,[48] and back extraction into sulfuric acid[3,48] or solid phase extraction using gauze columns.[49] The methods produced lower limits of detection of 30 ng/ml[48,49] and 20 ng/ml of plasma.[3]

5.2.2.23 Penbutolol

Penbutolol plasma concentrations have been determined by reverse phase chromatography at 28°C on a C_8 packed column using fluorescence detection[50] with a single extraction step followed by sample evaporation; the lower limit of detection was 2 ng/ml of plasma.

5.2.2.24 Pindolol

Plasma concentrations of pindolol have been determined using ultraviolet,[3,53] fluorescence,[51,54,55] and electrochemical detection.[52] Methods utilizing ultraviolet detection have been carried out on both C_{18} packed columns[3] and cyano packed columns[53] following a back extraction step into sulfuric acid. Limits of detection for the two methods were reported as 20 ng/ml and 1.2 ng/ml of plasma, respectively.

Electrochemical detection methods were used with a reverse phase chromatographic separation on a C_{18} packed column.[52] Back extraction into perchloric acid provided sufficiently clean samples to produce a lower limit of detection of 0.5 ng/ml of plasma, although no internal standard was used in this assay.

Fluorescence detection was used in three reported methods for analysis of pindolol in plasma.[51,54,55] In one method[51] the authors chose not to employ an internal standard method in order to minimize the probability of potential drug interferences. The other authors[54,55] also relied on measured transfers and injections instead of internal standard methodology.

5.2.2.25 Practolol

All reported assay methods for practolol concentrations in plasma have utilized ultraviolet detection.[2,56,57]

Reverse phase chromatography was used in all methods with C_{18} packed columns. A single extraction step followed by sample evaporation provided lower limits of detection of 50 ng/ml[2,56] and 30 ng/ml of plasma.[57]

5.2.2.26 Propranolol

A large variety of columns, column temperatures, extraction methods, mobile phases, internal standards, and fluorometer wavelength settings have

been used in the large number of reported high-performance liquid chromatographic assays for propranolol concentrations in plasma.[2,3,6,10,12,13,46,58-82] The reported lower limits of detection range from 0.2-20.0 ng/ml of plasma. The vast majority of reported assays utilized fluorescence detection. Ultraviolet detection after separation on a C_{18} packed column gave a lower limit of 20 ng/ml of plasma.[67]

Fluorescence detection following separation on C_{18} packed columns was reported by the largest number of authors[3,6,10,12,46,59,61-63,71,73,75,77,78,82] with separation on cyano columns following next.[2,64-66,72,79] The majority of column temperatures for C_{18} separation were reported as room temperature with others reported as, $40°C$,[82] $50°C$,[62] and $65°C$.[59] Column temperatures for separation on cyano columns were room temperature except for two assays at $35°C$,[3] and $55°C$.[72] Both single step extraction[2,6,45,58,61,64-69,74,75,80] and back extraction methods[3,10,59,60,62,63] were reported for sample preparation. Solid phase extraction on disposable C_{18} packed extraction cartridges was reported by several authors.[73,77,79]

Three methods involving chromatography with C_{18} packed columns, and one method using a cyano packed column, did not use an internal standard.[10,12,58,68]

Ion-pairing reagents were incorporated into the mobile phases of several assay methods.[10,61,68,72,77] Sodium heptanesulfonic acid was used most commonly.[61,68,72] Additionally, sodium pentanesulfonic acid[77] and sodium octyl sulfate[10] were used. The methods that utilized these reagents were designed to measure both propranolol and its active hydroxylated (4-hydroxy propranolol) metabolite, and the added reagents provided the necessary selectivity.

Lower limits of detection reported from C_{18} packed columns ranged from 0.2-6 ng/ml, and for cyano packed columns from 1.0-10 ng/ml of plasma.

Several authors reported methods using C_8 packed columns.[13,70] One method required several extractions with a final evaporation prior to chromatographic separation at $40°C$[70] to give a lower limit of 1 ng/ml of plasma. Another method involved a single extraction step followed by derivitization with acetic anhydride and separation at room temperature to give a lower limit of 1 ng/ml of plasma.[13]

Chromatographic separation on phenyl packed columns was reported by two investigators,[60,80] with both methods producing a reported lower limit of 1 ng/ml of plasma. A method[74] was reported that used a TSK-Gel LS 410 packed column with sample workup involving single extraction and evaporation steps with a lower limit of 0.5 ng/ml. The sample residue was reconstituted prior to injection with methanol containing pindolol as "internal standard" but adding the pindolol at that point does not truly represent the use of an internal standard method.

Chromatography on a poly(styrene-divinyl benzene) packed column at 30°C has been reported.[81] This method used a single extraction step and produced a lower limit of detection of 1 ng/ml of plasma.

A Pirkle Type A column was used in a method requiring a derivitization step for the determination of propranolol enantiomers.[76] The method provides a lower limit of detection of 0.5 ng/ml.

5.2.2.27 Sotalol

Both ultraviolet[84,85] and fluorescence[6,83,86] detection has been used in the measurement of plasma concentrations of sotalol.

Ultraviolet detection from reverse phase chromatography on either a C_{18} packed column[85] or a phenyl packed column[84] was carried out following a single extraction step with sample evaporation. The value of the reported chromatographic method with the C_{18} column[85] is limited due to a lack of internal standard. The lower limit of detection for the chromatographic method using a phenyl column was 8 ng/ml of plasma.

Chromatographic separation was accomplished on C_{18} packed columns for all methods reporting the use of fluorescence detection. One reported method[83] used a precipitation step followed by two extractions for sample clean up to provide a lower limit of detection of 20 ng/ml. A single extraction step followed by sample evaporation was sufficient to provide a limit of 25 ng/ml in another reported method.[6] Solid phase extraction on C_{18} packed extraction cartridges enabled the quantification of as little as 10 ng/ml of plasma.[86]

Three assay methods used ion-pairing reagents in the mobile phase.[83-85] The reagents were heptanesulfonic acid,[83] octyl sodium sulfate,[84] and dodecyl sodium sulfate.[85]

5.2.2.28 Timolol

Ultraviolet[3,89] and electrochemical[87] detection methods have been used in the measurement of plasma concentrations of timolol. Ultraviolet detection following reverse phase chromatography on C_{18} packed columns have reported detection limits of 40 ng/ml[3] and 2 ng/ml.[88] The second method provides as much sensitivity as another method employing more extensive sample clean up and electrochemical detection.[87]

5.2.3 DISCUSSION

The primary method of detection for the majority of beta-blocking agents is fluorescence detection. Some beta-blockers, such as oxprenolol, practolol, and bupranolol[24] are incapable of sufficient fluorescence and are more suited for detection by ultraviolet means. In general, fluorescence provides greater sensitivity and specificity (due to control of both

excitation and emission wavelengths) than ultraviolet detection and this is reflected in lower limits of detection. Some beta-blockers have been analyzed by electrochemical detection (bopindolol, mepindolol, pindolol, timolol) and, although electrochemical detection is very sensitive and can provide lower limits of detection in the picogram range, not all beta-blockers can produce sufficiently high signals to make this method useful. In addition, extensive work-up is necessary to produce clean samples, making this method less desirable for routine use.

The majority of the analytical methods described utilized reverse-phase chromatography. Very few methods reported a purely normal phase system while one group reported a method that used plain silica columns with a "reverse-phase" solvent system.[13]

The most common column packing for HPLC analysis was 5-10 micron C_{18} bonded silica. To achieve a desired selectivity, particularly when analyzing several beta-blockers or parent drug and metabolites, many authors reported using cyano, C_8, or phenyl bonded phases. Columns packed with spherical, macroporous poly(styrene-divinyl benzene) have also been used.[32,45,81] These columns are stabile across a wide range of pH and can be used to increase detectability when a beta-blocking agent has a fluorescence maximum at a pH extreme. The chromatographic columns were generally operated at ambient temperatures. Some groups reported the use of increased temperatures (up to 65°C) to shorten run-times or improve peak separations.[17,21,31,37,41,48,50, 55,59,62,65,70-74, 76,81,82]

The mobile phases used in the chromatography of the beta-blocking agents were most commonly a combination of acetonitrile, methanol, or both, with acetate or phosphate buffer. The pH of the eluting mobile phase was generally between 3.0-5.5. Some groups reported using higher pH in order to analyze the beta-blocker at its fluorescence maximum.[7,30,32] This was accomplished in one method[30] where the beta-blocker, labetalol, was separated on the column at a pH of 4.5 but detected at a pH of 9.3 by use of post-column alkalinization using a second solvent pumping system.

Tetrahydrofuran,[17,31,32] isopropranolol,[55,58,76,82] and ethanol[81] were also used in combination with acetonitrile or methanol as organic modifiers.

Ion-pairing agents have been used in many methods to alter the retention characteristics of individual beta-blockers, thus changing or improving peak symmetry, run time, and column selectivity. These methods can be particularly useful in resolving the drug peak from the peaks of endogenous substances or drug metabolites. The agents used include, heptane sulfonic acid,[9,34,37,42,61,68,70,83] dodecyl sodium sulfate,[10,35,49,85] octane sulfonic acid,[31,47,48,84] and pentane sulfonic acid.[77]

Another common method used to improve peak height and peak symmetry was the addition of small amounts of amine modifiers to the mobile phase to cap

exposed silica sites on bonded phase columns, thereby reducing peak tailing. Triethylamine was the amine most commonly used,[8,11,16,39,80,86] followed by diethylamine,[7,46] tetramethylenediamine,[17] dimethyloctylamine,[44] and n–butylamine.[82]

The extraction of the beta–blocking agents from plasma was generally achieved following alkalinization of the plasma and extraction into an organic phase. One study[3] examined different organic solvents and their ability to extract a number of beta–blocking agents. These investigators determined, on the basis of percent extracted, that a 60:20 combination of chloroform and pentanol was the best extraction solvent.

Ethyl acetate, diethyl ether, dichloromethane, and hexane with isoamyl or butyl alcohol have been used extensively as extracting solvents. Following extraction, the organic phase was either evaporated or back–extracted into a small volume of dilute acid. Back–extraction allows for reduction in contaminants while concentrating the beta–blocker and internal standard in a small volume that is ready for injection. Solid phase, disposable extraction columns packed with C_{18} or cyano bonded phase packing have also been used for plasma extraction.[11,12,21,45,77,78,86] These methods can provide effective sample clean–up to give low limits of detection.

High–performance liquid chromatography methods have been used to quantitate the lipophilicity of beta–adrenergic blockers.[91] At the mobile phase pH of 7.4 in a reverse–phase system, the retention times for beta–blockers increase as lipophilic character increases. When retention times are plotted versus partition coefficients determined with octanol:water at $37°C$ and physiological pH, the correlation is highly significant (r=0.92). Thus, in this system, the highly lipophilic beta–blocker, propranolol, has a retention time of 16.2 minutes while the much less lipophilic beta–blocker, sotalol, has a retention time of 3.2 minutes. Using this system to determine the retention time of newer beta–blockers could provide useful information about which extraction methods and mobile phase constituents will be most useful in the work–up of a new assay method.

The majority of reported methods of HPLC analysis of beta–blocking agents incorporates an internal standard method for determining concentration. A number of assays did not use internal standard methods but relied on measured transfers during sample handling, extraction and injection. The disadvantages of a method without an internal standard have been documented elsewhere.[89,90] The reliability suffers in the reported beta–blocker assay methods which do not utilize an internal standard.

Many authors have investigated the potential interference of other medications that might be coadministered with beta–adrenergic blockers. For instance, hydrochlorothiazide, hydralazine, alpha–methyldopa, furosemide, procainamide, quinidine, disopyramide, lidocaine, acetaminophen, diazepam,

and chlordiazepoxide. Few instances were reported of interferences, either because of a lack of UV or fluorescence activity at the given wavelengths, low extraction yield under the extraction conditions, or because of sufficiently different retention times from the beta-blocker in question and the assay internal standard. Yee and coworkers[9] reported interference from mexiletine with their atenolol assay. Shields and associates[53] determined that quinidine, n-acetylprocainamide, and lidocaine would interfere with the analysis of pindolol due to similar retention times under the assay conditions. The metabolites of verapamil were found to interfere with the analysis method of Piotrovskii and associates.[46]

A simple general scheme for determining plasma concentrations of a majority of beta-blockers would incorporate:

Sample Work-up
1)   addition of a suitable internal standard;
2)   plasma alkalinization;
3)   extraction into an organic phase;
4)   back-extraction into a small volume of dilute acid.

Chromatography
1) C_{18} column at ambient temperature;
2)   Mobile phase consisting of acetonitrile or methanol mixed with acetate or phosphate buffer at a pH of 3.0-5.5. The mobile phase could contain an amine modifier in a low concentration (0.1-0.4%) and a low concentration of ion-pairing agent, if needed to increase the lipophilicity, and thus retention time, of hydrophilic beta-blockers;
3)   Fluorescence detection.

Using this general format and substituting ultraviolet detection when necessary (for instance, for bupranolol, oxprenolol, and timolol) enables HPLC analysis of all beta-blocking agents. Changes in column temperature, mobile phase pH and organic modifier concentration can improve sensitivity and selectivity in many methods.

5.2.4   ACKNOWLEDGEMENTS

172

5.2.4 REFERENCES

1   T.W. Guentert, G.M. Wientjes, R.A. Upton, D.C. Combs and S. Riegelman,
     J. Chromatogr., 163 (1979) 373-382.
2   J.E. Holt, C.M. Kaye and M.G. Sankey, Br. J. Clin. Pharmacol., 12
     (1981) 282.
3   M.A. LeFebvre, J. Girault and J.B. Foutillan, J. Liq. Chromatogr., 4
     (1981) 483-500.
4   J.N. Buskin, R.A. Upton, R.M. Jones and R.L. Williams, J. Chromatogr.,
     230 (1982) 438-442.
5   M.G. Sankey, A. Gulaid and C.M. Kaye, J. Pharm. Pharmacol. 36 (1984)
     276-277.
6   R.M. Arendt, D.J. Greenblatt, R.H. deJong, J.D. Bonin and D.R.
     Abernethy, Cardiology, 71 (1984) 307-314.
7   I. Wiedemann, Arzneim. Forsch., 33 (1983) 861-864.
8   G.S.M.J.E. Duchateau, W.M. Albers and H.H. Van Rooij, J. Chromatogr.,
     382 (1986) 212-217.
9   Y.-G. Yee, P. Rubin and T.F. Blaschke, J. Chromatogr., 171 (1979)
     357-362.
10   H. Winkler, W. Ried and B. Lemmer, J. Chromatogr., 228 (1982) 223-234.
11   C. Verghese, A. McLeod and D. Shand, J. Chromatogr., 275 (1983)
     367-375.
12   P.M. Harrison, A.M. Tonkin, and A.J. McLean, J. Chromatogr., 339 (1985)
     429-433.
13   K.U. Bühring and A. Garbe, J. Chromatogr., 382 (1986) 215-224.
14   L.G. Miller and D.J. Greenblatt, J. Chromatogr., 381 (1986) 201-204.
15   H. Caqueret and G. Bianchetti, J. Chromatogr., 311 (1984) 199-305.
16   M. Canal and B. Flouvat, J. Chromatogr., 342 (1985) 212-215.
17   A. Darmon and J.P. Thernot, J. Chromatogr., 374 (1986) 321-328.
18   R.K. Bhamra, A.E. Ward, D.W. Holt, J. Chromatogr., 417 (1987) 229-232.
19   A. Selen, A.W. Kinkel, A.C. Darke, D.S. Greene and P.G. Welling, Eur.
     J. Clin. Pharmacol., 30 (1986) 699-704.
20   C.J. Oddie, G.P. Jackman and A. Bobik, J. Chromatogr., 273 (1983)
     469-474.
21   H. Humbert, J. Denouel, and H.P. Keller, J. Chromatogr., 422 (1987)
     205-215.
22   P. Haelfelfinger, J. Chromatogr., 221 (1980) 327-335.
23   A. Nagakura and H. Kohei, J. Chromatogr., 232 (1982) 137-143.
24   H. Winkler and B. Lemmer, J. Chromatogr., 309 (1984) 193-197.
25   J.P. Jeanniot, G. Houin, P. Ledudal, D. Berthet, D. Lusseau, P. Gros
     and J.P. Tillement, J. Chromatogr., 278 (1983) 301-309.
26   K. Reiff, J. Chromatogr., 413 (1987) 355-362.
27   J.N. Buskin, R.A. Upton, F. Sörgel, R.L. Williams, E. Lang, and L.Z.
     Benet, J. Chromatogr., 230 (1982) 454-460.
28   A.L. Sintetos, J. Hulse and E.L.C. Pritchett, Clin. Pharmacol. Ther.,
     41 (1987) 112-117.
29   L.J. Dusci and L.P. Hackett, J. Chromatogr., 175 (1979) 208-210.
30   B. Oosterhuis B, M. Van Den Berg and C.J. Van Boxtel, J. Chromatogr.,
     226 (1981) 259-265.
31   P.A. Meredith, D. McSharry, H.L. Elliott and J.L. Reid, J. Pharmacol.
     Meth., 6 (1981) 309-314.
32   K.B. Alton, F. Leitz, S. Bariletto, L. Jaworsky, D. Desrivieres and J.
     Patrick, J. Chromatogr., 311 (1984) 319-328.
33   I.J. Hidalgo and K.T. Muir, J. Chromatogr., 305 (1984) 222-227.
34   H. Hengy and E. Kölle, J. Chromatogr., 338 (1985) 444-449.
35   E.M. Bargar, J. Chromatogr., 417 (1987) 143-150.
36   W. Krause, J. Chromatogr., 181 (1980) 67-75.
37   M.T. Rosseel, F.M. Belpaire, I. Bekaert and M.G. Bogaert, J. Pharm.
     Sci., 71 (1982) 114-115.
38   D.B. Pautler and W.J. Jusko, J. Chromatogr., 228 (1982) 215-222.
39   M.S. Lennard and J.H. Silas, J. Chromatogr., 272 (1983) 205-209.
40   G.D. Johnston, A.S. Nies, and J. Gal, J. Chromatogr., 278 (1983)
     204-208.

41   J.B. Lecaillon, J. Godbillon, F. Abadie and G. Gosset, J. Chromatogr.,
     305 (1984) 411–417.
42   F.M. Gengo, M.A. Ziemniak, W.R. Kinkel and W.B. McHugh, J. Pharm. Sci.,
     73 (1984) 961–963.
43   G. Pflugman, H. Spahn and E. Mutschler, J. Chromatogr., 421 (1987)
     161–164.
44   K. Balmer, Y. Zhang, P. Lagerström and B. Persson, J. Chromatogr., 417
     (1987) 357–365.
45   R.N. Gupta, R.B. Haynes, A.G. Logan, L.A. MacDonald, R. Pickersgill and
     C. Achber, Clin. Chem., 29 (1983) 1085–1087.
46   V.K. Piotrovskii, V.G. Beloipetskaya, A.R. El'man and V.I. Metelitsa,
     J. Chromatogr., 278 (1983) 469–474.
47   C.D. Kinney, J. Chromatogr., 305 (1984) 489–495.
48   S.E. Tsuei, J. Thomas and R.G. Moore, J. Chromatogr., 181 (1980)
     135–140.
49   F. Plavsic, Acta Pharm. Jugosl., 32 (1982) 137–140.
50   N. Bernard, G. Cuisinaud and J. Sassard, J. Chromatogr., 228 (1982)
     355–361.
51   M. Bangah, G. Jackman and A. Bobik, J. Chromatogr., 183 (1980) 255–259
52   B. Diquet, J.J. Nguyen-Huu and H. Boutron, J. Chromatogr., 311 (1984)
     430–433.
53   B.J. Shields, J.J. Lima, P.F. Binkley, C.V. Leier and J.J. MacKichan,
     J. Chromatogr., 378 (1986) 163–171.
54   P.-H. Hysu and K.M. Giacomini, J. Pharm. Sci., 75 (1986) 601–605.
55   H.T. Smith, J. Chromatogr., 415 (1987):93–103.
56   M.J. Cooper and B.L. Mirkin BL, J. Chromatogr., 163 (1979) 244–246.
57   A.C. Mehta and R.T. Calvert, J. Chromatogr., 276 (1983) 208–212.
58   W.D. Mason, E.N. Amick and O.H. Weddle, Anal. Lett., 10 (1977) 515–521.
59   G.J. Schmidt and F.L. Vandemark, Chrom. Newslet., 5 (1977) 42–44.
60   R.L. Nation, G.W. Peng and W.L. Chiou, J. Chromatogr., 145 (1978)
     429–436.
61   A.M. Taburet, A.A. Taylor, J.R. Mitchell, D.E. Rollins and J.L. Pool,
     Life Sci., 24 (1979) 209–218.
62   P. Jatlow, W. Bush and H. Hochster, Clin. Chem., 25 (1979) 777–779.
63   D.W. Schneck, J.F. Pritchard and A.H. Hayes, Res. Comm. Chem. Path.
     Pharmacol., 24 (1979) 3–11.
64   G. Nygard, W.H. Shelver and S.K.W. Khalil, J. Pharm. Sci., 68 (1979)
     379–381.
65   M. Simon and M. Babich-Armstrong, J. Anal. Toxicol., 3 (1979) 246–252.
66   M. Simon and R. Terry, Ther. Drug Mon., 1 (1979) 265–276.
67   L.P. Hackett and L.J. Dusci, Clin. Toxicol., 15 (1979) 63–66.
68   P. Gyselinck, J.P. Remon, R. Van Severn and P. Braeckman, Br. J. Clin.
     Pharmacol., 10 (1980) 406–407.
69   O.H. Drummer, J. McNeil, E. Pritchard and W.J. Louis, J. Pharm. Sci.,
     70 (1981) 1030–1032.
70   M.T. Rosseel and and M.G. Bogaert, J. Pharm. Sci., 70 (1981) 688–689.
71   M. Lo, B. Silber and S. Riegelman, J. Chromatogr. Sci., 20 (1982)
     126–131.
72   F. Albani, R. Riva and A. Baruzzi, J. Chromatogr., 228 (1982) 362–365.
73   K.A. Parrott, J. Chromatogr., 274 (1983) 171–178.
74   H. Takei, H. Ogata and A. Ejima, Chem. Pharm. Bull., 31 (1983)
     1392–1394.
75   M. Divoll, D.J. Greenblatt and R.M. Arendt, Int. J. Clin. Pharmacol.
     Ther. Toxicol., 22 (1984) 457–460.
76   I.W. Wainer, T.D. Doyle, K.H. Donn and J.R. Powell, J. Chromatogr., 306
     (1984) 405–411.
77   K. Ray, W.G. Trawick and R.E. Mullins, Clin. Chem., 31 (1985) 131–134.
78   P.M. Harrison, A.M. Tonkin, C.M. Cahill and A.J. McLean, J.
     Chromatogr., 343 (1985) 349–358.
79   A. El-Yazigi and C.R. Martin, Clin. Chem., 31 (1985) 1196–1197.
80   R.P. Koshakji and A.J.J. Wood, J. Pharm. Sci., 75 (1986) 87–89.
81   Y. Yamamura, K. Uchino, H. Kotaki, S. Isozaki and Y. Saitoh, J.
     Chromatogr., 374 (1986) 311–319.

174

82   E.C. Kwong and D.D. Shen, J. Chromatogr., 414 (1987) 365–379.
83   M.A. LeFebvre, J. Girault, M.C. Saux and J.B. Fourtillan, J. Pharm.
     Sci., 69 (1980) 1216–1217.
84   S. Kärkkäinen, J. Chromatogr., 336 (1984) 313–319.
85   B. Lemmer, T. Ohm and H. Winkler, J. Chromatogr., 309 (1984) 187–192.
86   M.J. Bartek, M. Vekshteyn, M.P. Boarman and D.G. Gallo, J. Chromatogr.,
     421 (1987) 309–318.
87   M.R. Gregg and D.B. Jack, J. Chromatogr., 305 (1984) 244–249.
88   M.S. Lennard and S. Parkin, J. Chromatogr., 338 (1985) 249–252.
89   S.R. Bakalyar, Principles of Liquid Chromatography, in P.M. Kabra and
     L.J. Marton (Editors), Liquid Chromatography in Clinical Analysis,
     Humana Press, Inc., Clifton, NJ, 1981, p 3–19.
90. S.H. Wong, Drugs and Laboratory Management: Clinical and Technical
     Considerations, in S.H. Wong (Editor), Therapeutic Drug Monitoring and
     Toxicology by Liquid Chromatography, Marcel Dekker, New York, NY, 1985.
91   R.M. Arendt and D.J. Greenblatt, J. Pharm. Pharmacol., 36 (1984)
     400–401.

5.2.5 APPENDIX: SUMMARY OF METHODS FOR QUANTITATION OF BETA BLOCKERS

	Column	Detection	Extraction	IS	Lower Limit	Met.
Acebutolol						
Guentert, et al., 1971	C_{18}	UV, 240	Extract/Back-extract	[1] ——	10 ng/ml	Yes
Holt, et al., 1981	C_{18}	F 254/460	Extract/Evap	[2] MB 17, 764	10 ng/ml	No
LeFebvre, et al., 1981	C_{18}	F 235/389	Extract/Back-extract	LM 5008	5 ng/ml	Yes
Buskin, et al., 1982	C_{18}	UV, 243	Extract/Back-extract	[1] ——	20 ng/ml	Yes
Sankey, et al., 1984	C_{18}	F 254/418	Extract/Derivitize[3] Extract/Evap	[2] MB17, 764	50 ng/ml	Yes
Arendt, et al., 1984	C_{18}	UV, 235	Extract/Evap	MB-18973A	50 ng/ml	No
Adimolol						
Wiedmann, 1983	C_{18}	F 289/341	Extract/Back-extract	MEN-1098	0.5 ng/ml	No
Alprenolol						
Duchateau, et al., 1986	C_{18}	F 200/300	Extract/Back-extract	Pyrimethamine	0.33 ng/ml	No

(1) = (±)-(-(2-propryl-4-n-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane
(2) a propionamido analog of acebutolol
(3) with (S-(-)-N-trifluoroacetylprolyl chloride
Abbreviations: UV = ultraviolet
                F = fluorescence
                EC = electrochemical
                IS = internal standard
              Met. = metabolites

APPENDIX (continued)

	Column	Detection	Extraction	IS	Lower Limit	Met.
Atenolol						
Yee, et al., 1979	C_{18}	F 212/226	Extract/Back-extract	Metoprolol	2 ng/ml	No
LeFebvre, et al., 1981	C_{18}	F 222/no emission filter	Extract/Back-extract	Procainamide	10 ng/ml	No
Winkler, et al., 1982	C_{18}	F 280/300	Extract/Back-extract	None	2 ng/ml	No
Verghese, et al., 1983	CN	UV, 224	CN Extract Cartridge	Practolol	10 ng/ml	No
Harrison, et al., 1985	CN	F 193/ No emission filter	CN Extract Cartridge	None	10 ng/ml	No
Bühring and Garbe, 1986	Si	F 225/300	Extract/Evap/Recon/ Extract/Evap	Metoprolol	5 ng/ml	No
Miller and Greenblatt, 1986	C_{18}	F 280/300	Extract/Evap	Metoprolol	5 ng/ml	No

	Column	Detection	Extraction	I.S.	Lower Limit	Met.
Betaxolol						
Caqueret & Bianchetti, 1984	CN	F 275/305	Extract/Evap	Metoprolol	1 ng/ml	No
Canal & Flouvat, 1985	C_{18}	F 270/320	3 Extract/Evap	4-methyl-propranolol	4 ng/ml	No
Darmon & Theret, 1986	[1]C_{18}	F 285/330[2]	Extract/Evap/ Derivitize/Evap Recon/Evap/Recon	s(−)-ciclo-prolol	0.5 ng/ml	No
Bhamra, et al, 1986	Si	F 195/No emission filter	Extract/Evap	benzimida-zole	5 ng/ml	No
Bevantolol						
Selen et al, 1986	CN	UV, 205	Extract/ Back-Extract	pronethalol	26 ng/ml	Yes
Bisoprolol						
Bühring & Garbe, 1986	Si	F 225/300	Extract/ Back-Extract	[3] —	1-2 ng/ml	No

(1) Column temperature = 36°C
(2) R(−)-1-(1-Naphthyl)ethylisocyanate
(3) 1-[p-(tetrahydro-3-furanyl)phenoxy]-3-isopropylamino-2-propanol hemifurate

	Column	Detection	Extraction	I.S.	Lower Limit	Met.
Bopindolol						
Oddie et al, 1983	CN	F 220/320	Extract[1]	None	0. ng/ml	Yes
Humbert et al, 1987[3]	[5]C_{18}	EC	C_{18} Extract Cartridge	Mepindolol	0.05 ng/ml	Yes
Bufuralol						
Haefelfinger, 1980	C_{18}	F 250/300	Extract/Extract/ Wash/Extract/Evap	Desmethyl-bufuralol	5 ng/ml	No
Bunitrolol						
Nagakura & Kahei, 1982	[3]C_{18}	F 295/330	Extract/Backextract	Propranolol	2.5 ng/l	Yes
Bupranolol						
Winkler & Lemmer, 1984	CN	UV 200	Extract/Evap	None	1 ng/ml	No
Butofilolol						
Jeanniot et al, 1983	C_{18}	UV, 313	Extract/Evap	CM6859	20 ng/ml	No

(1) Enzyme hydrolysis to active metabolite. Bopindolol calculated from amount of metabolite measured with and without hydrolysis.
(2) Only measures hydrolyzed metabolites which is active component
(3) Column temperature = 40°C

	Column	Detection	Extraction	IS	Lower Limit	Met.
Carvedilol						
Reiff, 1987	C_{18}	F 285/340	Extract/Back-extract	Naftopidil	0.4 ng/ml	Yes
Celiprolol						
Buskin, et al., 1982	C_{18}	UV, 237	Extract/Back-extract	Acebutolol	10 ng/ml	No
Buskin, et al., 1982	C_{18}	F 335/472	Extract/Back-extract	Quinidine	5 ng/ml	No
Esmolol						
Sintetos, et al., 1987	C_{18}	UV, 229	Extract/Evap	None	50 ng/ml	No

	Column	Detection	Extraction	IS	Lower limit	Met.
Labetalol						
Dusci and Hackett 1979	C_{18}	UV, 233	Extract/Evap	Pericyazine	40 ng/ml	No
Oosterhuis, et al., 1981	C_{18}	F 335/370	Extract/Evap	Chloroquine	1 ng/ml	No
Meredith, et al., 1981	[1]C_{18}	F 305/418	Extract/Evap	Prazosin	8 ng/ml	No
Alton, et al., 1984	[2]PRP-1	F 370/415	Extract/Back-extract	[3]	4 ng/ml	No
Hidalgo and Muir, 1984	C_{18}	UV, 216	Extract/Back-extract	Propranolol	10 ng/ml	No
Levobunolol						
Hengy and Kolle, 1985	C_{18}	F 225/295	Extract/Evap	Metoprolol	0.5 ng/ml	Yes
Medroxalol						
Bargar, 1987	C_{18}	F 228/3898	On-line column switching	MDL 17,466	50 ng/ml	No
Mepindolol						
Krause, 1980	C_{18}	EC	Extract/Back-extract/ Wash	pindolol	1 ng/ml	No

1 Column temperature = 30°C
2 Spherical, macroporous poly(styrene-divinyl benzene)
3 =5-{2-[4-(4-methylphenyl)-2-butylamino]-1-hydroxyethyl}salicylamide

	Column	Detection	Extraction	IS	Lower Limit	Met.
Metoprolol						
LeFebvre, et al., 1981	C_{18}	UV 280	Extract/Back-extract	[1] acebutolol metabolite	10 ng/ml	No
Rosseel, et al., 1982	[2]C_8	F 220/295	Extract/Back-extract	4-methyl-propranolol	5 ng/ml	No
Winkler, et al., 1982	C_{18}	F 280/300	Extract/Back-extract	None	2 ng/ml	No
Pautler and Jusko, 1982	Si	F 224/ no emission filter	Extract/Back-extract	[3] —	3 ng/ml	Yes
Lennard and Silas, 1983	C_{18}	F 222/320	Extract/Evap	[4] —	5 ng/ml	Yes
Johnston, et al., 1983	C_{18}	F 275/300	Extract/Evap	Alprenolol	2 ng/ml	No
Lecaillon, et al., 1984	[5]C_8	F 225/320	Extract/Back-extract	Alprenolol	10 ng/ml	Yes
Gengo, et al., 1984	C_{18}	F 230/300	Precip/Extract/Evap	Pronethalol	5 ng/ml	No
Harrison, et al., 1985	C_{18}	F 193/ no emission filter	C_{18} Extract Cartridge	None	2 ng/ml	No
Bühring and Garbe, 1986	Si	F 225/300	Extract/Evap/Recon/ Wash/Evap	Bisoprolol	1-2 ng/ml	No
Pflugmann, et al., 1987	C_{18}	F 265/313	Extract/Derivitize/ Evap[6]	None	2 ng/ml	No
Balmer, et al., 1987	C_{18}	F 228/306	Extract/Back-extract Wash	Pafenolol	5 ng/ml	Yes

(1) (±)-1-(2-acetyl-4-acetamidophenoxy)-2-hydroxy-3-isopropylaminopropane
(2) Column Temperature = 40°C
(3) (±)-ethyl-2-(4-(3-isopropyl-amino-2-hydroxy-propoxy)phenyl)-ethyl carbamate
(4) 1-(4-butyramido-2-butylryl-phenoxy)-2-hydroxy-3-isopropylaminopropane
(5) Column Temperature = 50°C
(6) With (S)-(-)-phenylethyl isocyanate

APPENDIX (Continued)

	Column	Detection	Extraction	IS	Lower Limit	Met.
Nadolol						
Gupta, et al., 1983	[1]PRP-1	F 265/305	C_{18} Extract Cartridge	SQ 23554	10 ng/ml	No
Piotroviskii, et al., 1983	C_{18}	F 205/No emission filter	Extract/Evap	Glaucin	1 ng/ml	No
Kinney, et al., 1984	C_{18}	UV, 220	Extract/Evap	Acebutolol	20 ng/ml	No
Oxprenolol						
Tsuei, et al., 1980	[2]C_{18}	UV, 275	Extract/Back-extract	Alprenolol	30 ng/ml	No
LeFebvre, et al., 1981	C_{18}	UV, 280	Extract/Back-extract [3]PL333		20 ng/ml	No
Plavsic, 1982	C_{18}	UV, 275	Solid Phase Extract	Alprenolol	30 ng/ml	No

(1) poly(styrene-divinyl benzene)
(2) column temperature = 30°C
(3) (diphenyl-1, 2-butyl-4[N-carbonyl-oxypiperazinyl-N(hydroxyethyl-2)]5-pyrazoline-4-one-3

	Column	Detection	Extraction	IS	Lower Lmit	Met.
Penbutolol						
Bernard, et al., 1982	$^{(1)}C_8$	F 278/310	Extract/Evap	Propranolol	2 ng/ml	Yes
Pindolol						
Bangah, et al., 1980	C_{18}	F 220/320-400	Extract/Back-extract Wash	None	4 ng/ml	No
LeFebvre, et al., 1981	C_{18}	UV, 280	Extract/Back-extract	Oxprenolol	20 ng/ml	No
Diquet, et al., 1984	C_{18}	EC	Extract/Back-extract	None	0.5 ng/ml	No
Shields, et al., 1986	CN	UV, 220	Extract/Back-extract	Alprenolol	1.2 ng/ml	No
Hsyu and Giacomini, 1986	C_{18}	F 209/320	Extract/Derivitize/ Evap$^{(2)}$	None	2 ng/ml	No
Smith, 1987	$^{(3)}C_8$	F 255/315	Extract/Back-extract	None	2 ng/ml	No
Practolol						
Cooper and Mirkin, 1979	C_{18}	UV, 254	Extract/Evap	None	50 ng/ml	No
Holt, et al., 1981	C_{18}	UV, 245	Extract/Evap	Diacetolol	50 ng/ml	No
Mehta and Calvert, 1983	C_{18}	UV, 248	$^{(4)}$—	Acebutolol	30 ng/ml	No

(1) Column temperature = 28°C
(2) With (S)-(-)-alpha-methylbenzyl isocyanate
(3) Column temperature = 60°C
(4) not reported

APPENDIX (Continued)

	Column	Detection	Extraction	I.S.	Lower Limit	Met.
Propranolol						
Mason et al, 1977	CN	F 220 No em-filter	Extract/Evap	None	2 ng/ml	Yes
Schmidt & Vandemark, 1977	$^{(1)}C_{18}$	F 293/375	Extract/Back- Extract/Extract Evap	Pro- triptyline	"not estab."	No
Nation et al, 1978	Phenyl	F 205/340	Extract/Back- Extract	4-methyl propranolol	1 ng/ml	Yes
Taburet et al, 1979	C_{18}	F 295/340	Extract/Evap	Pronethalol	1.5 ng/ml	No
Jatlow et al, 1979	$^{(2)}C_{18}$	F 285/350	Extract/Back- Extract	Pronethalol	6 ng/ml	No
Schneck et al, 1979	C_{18}	310/No emission filter	Extract/Back- Extract	Pronethalol	1 ng/ml	Yes
Nygard et al, 1979	CN	F 276/340	Extract/Evap	Cyclo- methycaine	10 ng/ml	No
Simon, Babich-Armstrong, 1979	$^{(3)}$CN	F 215/340	Extract/Evap	Protrip- tyline	5-10 ng/ml	No
Simon & Terry, 1979	CN	F 210/340	Extract/Evap	Pronethalol	10 ng/ml	No
Hackett & Dusci, 1979	C_{18}	UV, 230	Extract/Evap	Promazine	20 ng/ml	No
Gyselinck et al, 1980	C_{18}	F 254/360	Extract/Evap	None	Not reported	Yes
LeFebvre et al, 1981	C_{18}	F 215/No Em. filter	Extract/Back- Extract	LM 5008[4]	0.5ng/ml	No

(1) Column temperature = $65°C$
(2) Column temperature = $50°C$
(3) Column temperature = $35°C$
(4) 4-[2-(3-indolyl)-ethyl]piperidine

	Column	Detection	Extraction	I.S.	Lower Limit	Met.
Propranolol (con't)						
Drummer et al, 1981	C_{18}	F 310/380	Extract/Evap	Labetalol	2 ng/ml	Yes
Holt et al, 1981	CN	F 254/340	Extract/Evap	MB 19,421	2 ng/ml	No
Rosseel & Bogaert, 1981	[5]C_8	F 290/340	Extract/Back- Extract/Extract Evap	4-methyl- propranolol	1 ng/ml	Yes
Winkler et al, 1982	C_{18}	F 280/333	Extract/Back- Extract	None	2 ng/ml	No
Lo et al, 1982	[6]C_{18}	F 216/340	Extract/Back- Extract	ICI 45837	0.2 ng/ml	Yes
Albani et al, 1982	[7]CN	F 285/405	Precipitation	Desipramine	5 ng/ml	Yes
Parrott, 1983	C_{18}	F 280/330-380	C_{18} Extraction Cartridge	Propyl-paraben	1.8 ng/ml	No
Piotrovskii et al, 1983	C_{18}	F 225/No Em filter	Extract/Evap	Glaucin	1 ng/ml	No
Takei et al, 1983	[8]TSK-GEL LS 410	F 295/337	Extract/Evap	Pindolol[9]	0.5 ng/ml	Yes
Arendt et al, 1984	C_{18}	F 235/335	Extract/Evap	Pronethalol	1 ng/ml	No
Divoll et al, 1984	C_{18}	F 235/335	Extract/Evap	Pronethalol	0.5 ng/ml	No

(5) Column temperature = 40°C
(6) Column temperature = 30°C
(7) Column temperature = 55°C
(8) Column temperature = 24°C
(9) Was only added right before injection.

	Column	Detection	Extraction	I.S.	Lower Limit	Met.
Propranolol (con't)						
Wainer et al, 1984 [10]	Pirkle Type A	F 290/335	Extract/ Derivitization/ Evap [11]	Pronethalol	0.5 ng/ml	No
Ray et al, 1985	C_{18}	F 296/338	C_{18} Extract Cartridge	4-methyl-propranolol	5 ng/ml	No
Harrison et al, 1985	C_{18}	F 217/360	C_{18} Extract Cartridge	None	1 ng/ml	Yes
El-Yazigi & Martin, 1985	CN	F 250/336	Extract/Evap	Metoprolol	1 ng/ml	No
Koshakji & Wood, 1986	Phenyl	F 228/No Em. filter	Extract/Evap	N-ethyl-propranolol	1 ng/ml	No
Yamamura et al, 1986 [12]	PRP-1	F 285/340	Extract/Evap	Penbutolol	1 ng/ml	No
Bühring & Garbe, 1986	C_{8}	F 225/320	Extract/Evap/ Derivitize/ Evap [13]	Bisuprolol	1 ng/ml	No
Kwong & Shen, 1987 [14]	C_{18}	F 216/280	Multiple Extract	Pronethalol	3 ng/ml	Yes

(10) Column temperature = 20°C
(11) With phosgene to cyclic-2-oxazolidone derivative
(12) Column temperature = 30°C; styrene-divinyl benzene
(13) With acetic anhydride
(14) Column temperature = 40°C

	Column	Detection	Extraction	IS	Lower Limit	Met.
Sotalol						
LeFebvre, et al., 1980	C_{18}	F 235/No emmision filter	Precip/Extract/ Back-extract	Procainamide	20 ng/ml	No
Kärkkäinen, 1984	Phenyl	UV, 226	Extract/Evap	Atenolol	8 ng/ml	No
Lemmer, et al., 1984	C_{18}	UV, 227	Extract/Evap/ Recon/Wash	None	10 ng/ml	No
Arendt, et al., 1984	C_{18}	F 235/310	Extract/Evap	Pronethalol	25 ng/ml	No
Bartek, et al., 1987	C_{18}	F 240/310	C_{18} Extraction Cartridge	[1] —	10 ng/ml	No
Timolol						
LeFebvre, et al., 1981	C_{18}	UV, 295	Extract/Back-extract	[2] PL 333	40 ng/ml	No
Gregg and Jack, 1984	C_{18}	EC	Extract/Back-extract Wash	Propranolol	2 ng/ml	No
Lennard and Parkin, 1985	C_{18}	UV, 295	Extract/Evap	Phenacetin	2 ng/ml	No

(1) 4'-(2-cyclohexylamino-1-hydroxyethyl-methane sulfonanilide

(2) diphenyl-1,2-butyl-4[N-carbonyl-oxypiperazinyl-N(hydroxyethyl-2)]
-5-pyrazoline-4-one-3

Chapter 5.3

THIN-LAYER CHROMATOGRAPHY

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5.3.1 INTRODUCTION

Thin-layer chromatography (TLC) differs from most other analytical techniques in that it allows drug separation and identification without the use of expensive equipment. Furthermore in contrast to gas liquid chromatography (GLC) and high pressure liquid chromatography (HPLC) many samples can be run simultaneously. If a drug is essentially non-volatile and dissolves in organic solvents, it can be separated from other drugs or foreign materials simply by varying the plate material, the chromatographic system or the temperature. In addition, drug present on TLC plates can be detected using very sensitive reagents. Therefore this method is the technique of choice for drug screening (e.g. in proving an intoxication). Moreover qualitative TLC may be used in the evaluation of patient compliance. Several reports on the detection of β-adrenoceptor blocking drugs in urine have been published in the literature (refs. 1-4). Other reports have dealt with the use of TLC for the identification of these drugs in tablets, ampules etc. (refs. 5-7) and the evaluation of their purity (for review see ref. 8). In addition, the behaviour in various chromatographic systems of several hundreds of drugs (pure substances), including β-adrenoceptor blockers, has been described (refs. 9-11). For 11 β-adrenoceptor antagonists substituted with an isopropyl residue the dansylation (Fig. 5.3.1) and characterization by two-dimensional TLC has been described (ref. 12).

190
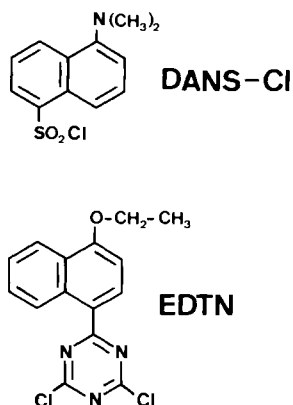
Fig. 5.3.1. Structures of the fluorescent reagents 5-dimethyl-amino-1-naphthalenesulfonyl chloride (DANS-Cl) and 1-ethoxy-4-(dichloro-s-triazinyl)-naphthalene (EDTN)

TLC can be also used to determine drug concentrations in biological material. As the concentrations of β-adrenoceptor antagonists in biological material, especially in plasma (serum, blood), are very low (usually in the ng-range) solvent extraction and evaporation has to be performed to increase the drug concentration in the extract. This serves also as a first purification step. TLC is used for the final separation of the respective drug from co-extracted impurities.

5.3.1.1 Thin-layer chromatography: equipment and methods

The principles of quantitative TLC as well as parameters important to obtain good reproducibility of the results have been discussed already (for review see refs. 13-18). Therefore in this article only the techniques relevant for the determination of β-adrenoceptor antagonists are mentioned.

For quantitative determinations it is essential, that the structure of the sorbent is homogeneous, as this strongly influences the size and form of the spots and therefore the reproducibility of the method (ref. 13). These days pre-coated plates are generally used. β-adrenoceptor blocking drugs are usually chromatographed on silica gel 60 coated glass plates (layer thickness 0.25 mm) without fluorescence indicator. Sometimes high performance thin-layer plates (HPTLC) are used instead of the normal ones (e.g. refs. 19, 20). The use of HPTLC plates can lead to a reduction in both average spot diameter and separation time and thus can increase sensitivity. This is due to sample zones more concentrated for a given migration distance (ref. 21). If, however, drug concentrations have to be determined in biological material, large amounts often have to be applied onto the plates and thus the advantage of HPTLC as compared to TLC is less marked.

For qualitative drug detection, fluorescence indicator containing plates can be used (refs. 11, 22, 23). For quantitative purposes, however, the measurement of fluorescence quenching is not useful (ref. 17). In contrast to the other β-adrenoceptor blocking drugs and their metabolites 4-hydroxy-propranolol is chromatographed using microcrystalline cellulose plates because silica gel, aluminium oxide and polyamide plates cause degradation of the sensitive metabolite (ref. 24).

Besides the structure of the adsorbent, the techniques used for spotting the samples onto the plates is important, if results are to be reproducible (refs. 13, 17). Nowadays this can be performed using micro pipettes, micro capillaries, Hamilton syringes or automatic spotting systems. The latter ones may apply either one sample at a time (e.g. Linomat III, Camag) or multiple samples simultaneously (e.g. Autospotter, Desaga; Multi-Spotter, Analytical Instrument Specialities). Usually applying the samples as thin lines as compared to spots leads to improved resolution (refs. 13, 17, 18). A similar effect can be obtained by using plates with pre-adsorbent zones (ref. 18).

In general, the separation of drugs from interfering material co-extracted from biological specimens is performed by ascending chromatography in saturated glass chambers. Sometimes unsaturated ones are used. Due to the basic character of the β-adrenoceptor antagonists, usually small amounts of ammonia are added

to the developing solvents or the plates are developed in an at-
mosphere of ammonia (by putting beakers containing concentrated
ammonium hydroxyde solution into the glass tank). The solvents
for the chromatography of bunolol, carteolol (and metabolites),
nadolol and pindolol, as well as those for the metabolites of
propranolol, however, contain acids (refs. 24-30). The derivati-
ves of β-adrenoceptor blockers obtained by the reaction with
dansyl chloride, EDTN and NEIC (see below) are chromatographed
in neutral solvents (refs. 12, 19, 31, 32). The plates are deve-
loped for 5-19.5 cm depending on the respective drug. After a
drying period – and possible fluorescence enhancement by dipping
the plate into a paraffin containing solvent (see below) – the
plates are scanned.

In the lower concentration range light absorption of drugs in
cuvettes is linearly related to drug concentration (Lambert-
Beer-law). On the other hand there is a more complicated relati-
onship between light absorption and drug concentration on TLC
plates (Kubelka-Munk-function) for which, in addition, several
critical parameters exist (for review see refs. 14, 16, 17).
Drug fluorescence in cuvettes and on TLC plates, however, is di-
rectly proportional to the respective concentration/amount
(refs. 15, 17). For the determination of drugs in biological ma-
terial by TLC, however, the theoretical equations relating drug
amount and light absorption/fluorescence intensity are not used.
Instead, calibration curves are established for each plate by
including spiked samples. Alternatively, amounts of pure sub-
stance, dissolved in an organic solvent (e.g. methanol) may be
applied to separate starting lines on each plate. The drug reco-
very should then be determined on each working day by carrying a
lower number of spiked samples through the analysis. In general
the reproducibilities of these methods are considerably better
than of those using standard graphs obtained on separate plates
(possibly even on separate days (ref. 15)). For the quantitation
of the drug amounts on TLC plates either peak height or peak
area are used, these values being determined either graphically
or using an integrator. Sometimes an internal standard is added
to the samples. However, this is not as necessary as for example
in GLC.

Quality control of the analysis procedure can be performed by
preparing spiked plasma (or urine) samples by using separately

prepared stock solutions. Several of these samples (e.g. one per plate) are included in the analysis and their drug concentration is calculated. The result is compared to the theoretical concentration. Above that, whenever possible, spiked samples should be exchanged with other laboratories performing the analytics of the same drug.

5.3.1.2 Quantitative analysis by measurement of native fluorescence

In this method the chromatographic separation is followed by the quantitation of the drug. Fluorimetric determination directly on the TLC plate is the method of choice in case of a sufficiently strong native fluorescence of the β-adrenoceptor antagonist. Such methods have been published for the determination of acebutolol (refs. 33, 34), atenolol (refs. 35, 36), nadolol (ref. 29), penbutolol (refs. 20, 37), pindolol (ref. 30) and propranolol (refs. 24, 36, 38). In some cases, metabolites can be determined simultaneously (refs. 33, 34, 38). In another publication the fluorimetric determination of bufuralol has been described. The drug is separated from impurities and metabolites by TLC, then the native fluorescence of this drug is measured in a cuvette after elution from the silica gel with diluted hydrochloric acid (ref. 39). In the early years of TLC such a procedure was more common due to the lack of suitable scanning devices.

Frequently the native fluorescence of β-adrenoceptor antagonists and other drugs on TLC plates can be enhanced by spraying the plate with water/ethylene glycol or propylene glycol (e.g. refs. 24, 38) or by dipping the plate into paraffin containing solutions (refs. 28, 29, 31, 34, 37). The physico-chemical parameters of solvents relevant for fluorescence enhancement were investigated thoroughly studying the behaviour of DANS-amines (ref. 40).

Spraying the plate with water/ethylene glycol/citric acid was used for the determination of propranolol and atenolol. It was derived from the classical fluorimetric measurements in cuvettes. Measuring propranolol fluorescence in ethylene glycol/water/citric acid increased the fluorescence intensity more as compared to hydrochloric acid (ref. 41). Spraying the plate

with glycol/water, however, is critical. Fluorescence intensity
depends on the moisture content, thus care has to be taken to
ensure even spraying. In addition, since the water evaporates
rather rapidly, only a few minutes are left for scanning the
plate (refs. 24, 38). In view of this and the finding, that pro-
pranolol and atenolol fluorescence intensity are also improved
by dipping the plates into paraffin solutions (ref. 36), this
method is no longer used in our group.

5.3.1.3 <u>Absorbance reading</u>

    The determination of β-adrenoceptor antagonists by measuring
the absorbance of UV light may be necessary if there is no na-
tive fluorescence. Such a method has been described for the de-
termination of carteolol in plasma (ref. 26) and carteolol, 8-
hydroxy-carteolol and carteolol-glucuronide in urine (ref. 27).
UV absorbance is also suitable for the measurement of proprano-
lol in plasma (refs. 42, 43). The limit of detection, however,
is higher as in the case of fluorescence reading.

5.3.1.4 <u>Formation of fluorescing derivatives</u>

    The measurement of UV absorbance can frequently be avoided by
forming fluorescing derivatives of β-blockers. For TLC determi-
nation of these drugs 1-ethoxy-4-(dichloro-s-triazinyl)naph-
thalene (EDTN, Fig. 5.3.1) proved to be a suitable reagent.
Derivatization is performed after drug extraction from the body
fluid and evaporation of the solvent. The fluorescing derivative
is chromatographed on silica gel 60 plates. This method is used
for the determination of tolamolol (ref. 31) and oxprenolol
(ref. 32). As with native fluorescence of β-adrenoceptor
blocking drugs, fluorescence intensity of EDTN derivatives can
be enhanced by dipping the plate into a paraffin containing
solution.
    Pindolol can be determined by derivatization with o-phthaldi-
aldehyde after extraction from biological material. The product
has an about 10.000fold higher fluorescence than the parent
drug. The fluorescence of the derivative is read in cuvettes
(ref. 44). Spraying pindolol on TLC plates with o-phthaldialde-
hyde does not, however, lead to a lower detection limit of pin-

dolol as compared to the native drug on TLC plates dipped into a paraffin solution. This is due to a comparable increase in fluorescence of partially interfering peaks (ref. 30).

Derivatization can be used also for the simultaneous determination of the R- and S-enantiomers of β-adrenoceptor antagonists. Such methods are useful, as the S-enantiomers of these drugs are about 100fold more active than the R-enantiomers (e.g. ref. 45) and the pharmacokinetics of the enantiomers may differ. TLC separation can be performed after derivatization with chiral reagents. For the determination of various β-adrenoceptor antagonists (pure substance) the use of R-(-)-1-(1-naphthyl)ethyl isocyanate (NEIC; Fig. 5.3.2) has been described (ref. 19). The renal excretion of metoprolol, oxprenolol and propranolol enantiomers can be determined simultaneously after derivatization with S-(+)-benoxaprofen chloride (BOP-Cl; Fig. 5.3.2; ref. 45). Both reagents form strongly fluorescing derivatives with the amino groups of β-blockers. The diastereomeric derivatives can be separated by TLC and HPLC (refs. 19, 46). Further information is given in Chapter 7.
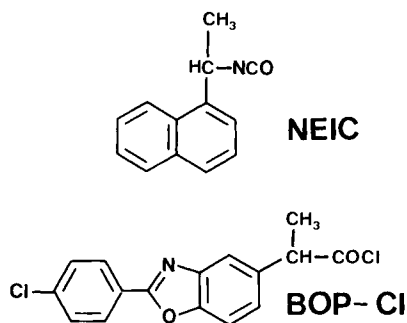
Fig. 5.3.2. Structures of the chiral derivatization reagents 1-(1-naphthyl)ethyl isocyanate (NEIC) and benoxaprofen chloride (BOP-Cl)

5.3.1.5 Scanning of radioactivity

TLC methods are frequently used for the evaluation of drug metabolism. For example, in animal experiments β-adrenoceptor

blockers containing radioactive isotopes (tritiated substances and [14]C-labelled ones) are administered, after which body fluids, particularly urine and plasma, as well as faeces are collected. The native drugs and their metabolites are then separated by TLC (and eventually paper chromatography) and the radioactivity is read directly on the plate or the adsorbant is scraped from the glass plates and placed in vials containing scintillation fluid (refs. 22, 23, 25, 28, 47). Due to ethical reasons, the use of radioactive labelled β-adrenoceptor antagonists is restricted to research studies. Therefore this chapter does not deal with these methods in detail.

In the following section, TLC methods for the determination of selected β-adrenoceptor antagonists are described.

5.3.2 DETERMINATION OF β-ADRENOCEPTOR ANTAGONISTS BY READING NATIVE FLUORESCENCE ON TLC PLATES

As stated above, in case of a drug which exhibits sufficiently strong native fluorescence the combination of TLC (or HPLC) and fluorescence reading is generally the method of choice. This is because impurities can only interfere if they have similar excitation and emission spectra to the drug being tested. In the following TLC methods for the determination of acebutolol, atenolol, bufuralol, nadolol, penbutolol and propranolol are described.

5.3.2.1 Acebutolol

The first method for the estimation of acebutolol concentrations in biological specimens by TLC-fluorimetry was described by Steyn (ref. 33). It was the first publication using TLC combined with in situ fluorescence reading for the estimation of the concentrations of a β-adrenoceptor blocking drug. Quinidine served as the internal standard. More recently another procedure was described by Möhrke and coworkers (ref. 34). Both methods allow the simultaneous determination of the major metabolite diacetolol.

Method I: 350 ng quinidine and 2 ml buffer solution pH 10 (carbonate/hydrogen carbonate; 0.2 M) are added to 2 ml serum. The mixture is shaken with 5 ml of ethyl acetate for 15 min. Af-

ter centrifugation, the ethyl acetate layer is transferred to another tube and evaporated at 50°C under a stream of nitrogen. The residue is then dissolved in 50 µl of methanol and 10 µl of the methanolic solution are spotted on silica gel 60 plates (using glass micropipettes). For calibration, 10 µl of a methanolic solution containing 400 ng acebutolol and diacetolol and 100 ng quinidine are spotted onto the plate. Separation of the drugs is performed for 5 cm with a freshly prepared solvent system consisting of ethyl acetate-methanol-ammonia (75:20:5) using unsaturated TLC chambers at room temperature. The plate is dried and heated in an oven at 90°C for 15 min. After cooling to room temperature it is dipped into a solution containing 10% paraffin wax in light petroleum and dried again. After a further 15 min it is scanned (excitation wave length: 350 nm; emission wave length: 450 nm). Drug concentrations in serum are determined from the peak-height ratio drug/internal standard related to the slope of a standard graph determined on a separate occasion. The slope of the standard graph is corrected for peak-height ratio of the control solutions calculated on the day of performing the standard graph and the day of analysis of the respective samples. Thus, contrary to most other procedures described for the TLC analysis of drugs calibration curves are not established for each plate.

The reproducibility of the method is nevertheless good (coefficient of variation about 4%). The extraction rates of acebutolol and diacetolol from serum were not determined, nor that of quinidine. Propranolol, practolol, sotalol, oxprenolol, alprenolol and pindolol were investigated for interference with the assay procedure, but none of them exhibits comparable fluorescence and the R_f values are in general higher.

Detailed investigations into the fluorescence behaviour of acebutolol and quinidine showed a rapid decline of acebutolol fluorescence due to the evaporation of ammonia from the plate after removing it from the tank. Consequently the fluorescence ratio acebutolol/quinidine decreased rapidly during the first hour after removing the plate. Heating the plate increased ammonia evaporation even more, with the result that the limit of acebutolol detection was too high for the determination of this drug in serum. Dipping the plate into the paraffin solution, however, increased the fluorescence intensity about eight-fold

and stabilized it for at least 48 h (ref. 33). Therefore covering the plate with paraffin was used in connection with the other method, too (ref. 34).

Method II: 0.2 ml 2N NaOH is added to 1 ml serum and extracted with 2.0 ml ethyl acetate. After centrifugation, 1 ml of the supernatant organic layer is transferred to another tube and evaporated to dryness at 60°C under nitrogen. The residue is dissolved in 50 µl ethyl acetate and 40 µl are spotted onto a silica gel plate. The chromatogram is developed in the solvent system chloroform-methanol (85:10) in an atmosphere of ammonia. After drying for 15 min the plate is dipped in a solution of 4% paraffin in cyclohexane. After another drying period the plate is scanned at an excitation wave length of 265 nm and an emission wave length of 436 nm.

From urine samples (1 ml) acebutolol and diacetolol are extracted into 0.5 ml ethyl acetate after addition of 0.2 ml 2N NaOH. After centrifugation 20 µl of the organic layer are spotted directly onto TLC plates.

Fig. 5.3.3 depicts the densitometer scans of blank serum, blank serum to which 500 ng/ml acebutolol and diacetolol have been added, and a patient serum obtained 3 h after the application of a 400 mg single dose of acebutolol. The limit of determination of both drugs in serum is 10 ng/ml. The coefficient of variation is 2.5%, 3.2% and 6.6% at 2000, 200 and 20 ng/ml, respectively. The calibration curve is linear up to 10 µg/ml. In urine no deviation of linearity is observed up to 100 µg/ml; the limit of determination is 200 ng/ml. The coefficient of variation is 2.5% at 20 µg/ml and 7% at 400 ng/ml. The method served for studying the pharmacokinetics of acebutolol in healthy young volunteers and elderly patients after single oral doses of 400 mg (ref. 34).

5.3.2.2 Atenolol

Atenolol can be estimated in plasma and urine by TLC-fluorimetry using methods similar to that described for acebutolol determination (refs. 35, 36). In the earlier publication a method for determination of atenolol in urine samples based on directly spotting 10 µl of urine onto TLC plates was described (ref. 35). This method is sufficient for the analysis of urine samples ob-

tained from healthy volunteers and moderately ill patients. Pro-
blems, however, arose when urine specimens of patients suffering
from severe renal failure were studied. An interfering peak was
often observed (ref. 48) which could be removed by extracting
atenolol into a small volume of butanol-cyclohexane from which
an aliquot is spotted onto the TLC plate. This modification
(ref. 36) is described below.
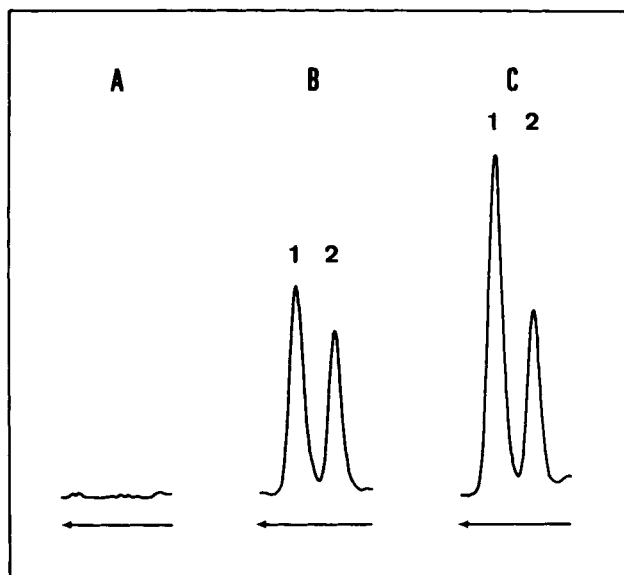
Fig. 5.3.3. Densitometer scans of blank serum (A), blank serum
to which 500 ng/ml acebutolol (1) and diacetolol (2) were added
(B), a patient serum obtained 3 h after the ingestion of a
single oral dose of 400 mg acebutolol (C). (From ref. 34 with
permission of the publisher)

Plasma: 0.5 ml 1N sodium hydroxide solution is added to 1 ml
plasma after which the drug is extracted for 15 min with 5 ml of
dichloromethan-butanol-(1) (95+5). The phases are separated by
centrifugation and the plasma is discarded, the organic layer
being transferred quantitatively into another tube. The solvent
is evaporated at 50°C under nitrogen and the residue is then
dissolved in 50 µl of methanol. 40 µl are spotted onto a silica
gel 60 plate without fluorescence indicator (width of the strip

8 mm) using a Linomat III. 100 ng atenolol are applied to three
starting lines per plate as calibration spots. The plate is de-
veloped in chloroform-methanol-glacial acetic acid (75:20:5) for
8 cm using standard conditions. The R_f value of atenolol is
0.16. After drying the plate is dipped into a solution contain-
ing 4% paraffin in cyclohexane and dried again. Atenolol fluo-
rescence is read directly on the TLC plate (excitation wave
length: 265 nm; emission wave length: 313 nm). The areas under
the peaks, calculated by peak height x width at half height, are
directly proportional to the atenolol amount/spot up to 2 μg. To
determine the content of unknown samples it is sufficient to de-
termine only one point on the calibration curve (mean of three
determinations) for each plate.

Urine: 0.5 ml 1N NaOH and 0.5 g NaCl are added to 1 ml urine
and the mixture is shaken with 0.4 ml cyclohexane-butanol for 15
min. After separation of the layer by centrifugation, 10 to 80
μl of the organic layer are spotted onto th TLC plate as descri-
bed above. 100 ng atenolol are applied to three starting lines
as calibration spots. The plate is developed in the solvent sy-
stem chloroform-methanol (88:12) in an atmosphere of ammonia for
8 cm (R_f value of atenolol is 0.20). Dipping of the plate, fluo-
rescence reading and calculation of atenolol are performed as
described for plasma.

The limits of atenolol determination are 5 ng/ml plasma and
25 ng/ml urine. The recovery rates amount to 68 and 72%,
respectively. The variation coefficients of the procedures are
about 5%. The method described here served for the determination
of atenolol pharmacokinetics in patients suffering from the im-
pairment of the kidney and liver function as well as for the in-
fluence of other drugs administered concomitantly on atenolol
kinetics (refs. 48-53).

5.3.2.3 Bufuralol

In 1976 de Silva and coworkers described a method for the si-
multaneous determination of bufuralol and its major metabolite,
the respective α-hydroxy-derivative. The procedure included a
TLC separation step to resolve the parent drug from the meta-
bolite. The silica gel areas containing the drugs are scraped
off and the drugs are eluted from the anorganic material and the

native fluorescence of bufuralol and its metabolite is read in cuvettes. The emission maxima are 300-305 nm (ref. 39). More recent investigations into atenolol, nadolol and pindolol, which have comparable fluorescence behaviours to bufuralol, showed also that drugs emitting rather short-wave fluorescence light can be quantitated directly on TLC plates (refs. 29, 30, 35, 36). Thus the extraction of bufuralol from the plate material does not seem to be essential. In the following, the procedure published by de Silva and coworkers is summarized.

The analysis is performed using siliconized glassware. 1.3 ml of 0.1N NaOH (1.5 ml of 1M borate-sodium carbonate-potassium chloride buffer pH 9.0) are added to 2.5 ml blood (urine). Bufuralol and its metabolite are extracted with 10 ml diethyl ether by shaking for 10 min. The phases are separated by centrifugation. The ether is transferred into another tube, the extraction is repeated once more and the extracts are combined. The drugs are re-extracted into an aqueous phase by adding 5 ml 0.1N HCl and shaking for 10 min. After centrifuging for 3 min the ether is discarded. The solution is made alkaline by adding 1N NaOH and extracted once more using two portions of 10 ml ether as described. The combined extracts are evaporated to dryness at 45°C. (Samples can be kept overnight, if they are vacuum dried and stored in a desiccator). The residues are dissolved in 100 µl of ether-methanol (90:10) and transferred quantitatively onto a silica gel 60 plate as a small point. Each tube is rinsed with 50 µl ether-methanol and the solution is combined with the original. On each plate the extracts of one control blood/urine and four separate specimens containing 25, 50, 75 and 100 ng/ml of the drugs are spotted along with the extracts of the unknown samples. Further specimens of 10 µg of the pure drugs are run alongside as standards.

The plates are developed for 12 cm in a vapour-saturated chamber containing methylene chloride-methanol-concentrated ammonium hydroxide solution (90:10:1). Silica gel areas (1.5 cm^2) corresponding to the R_f values of bufuralol (0.5) and its metabolite (0.25) are scraped off and transferred into separate centrifuge tubes. 1 ml borate-sodium carbonate-potassium chloride buffer pH 9.0 is added and the mixture is shaken on a vortex mixer for 30 sec. The slurry is extracted using two portions of 4 ml ether by shaking and centrifuging as described above. The

drugs are re-extracted from the combined ether phases into 2.5 ml 0.1N HCl and fluorescence of bufuralol (and the metabolite) is read in a 1-cm path quartz cell with excitation at 250 nm. The emission maximum is 305 (300) nm. The fluorescence readings of the standards and the unknown samples are corrected for the fluorescence of the blank sample. The concentrations of the standards serve for the construction of the calibration graphs for bufuralol and its metabolite respectively.

Investigations into the fluorescence behaviour of these drugs showed the same excitation maxima and slightly differing emission spectra. The relative fluorescence intensity of the two compounds measured at the respective emission maxima proved to be similar. The fluorescence response is linear from 10 to 3000 ng/ml. The determination of bufuralol concentrations below 10 ng/ml recovered from blood depends on the extensive sample clean up described above. Then the limit of detection is 2-4 ng/2.5 ml 0.1N HCl. The drugs are extracted quantitatively (>95%) from blood and urine as well as from the hydrochloric acid, if all glassware used for the procedure is deactivated by a siliconizing agent to minimize adsorption losses. The overall <u>recovery</u> of bufuralol extracted from blood and urine then is 86±3% and the recovery of the metabolite is 88±7%. The method proved to be sufficient for the determination of bufuralol and its metabolite in healthy volunteers following a single oral dose of 20 mg. Blood levels could be quantitated for 9 and 24 h respectively (ref. 39).

5.3.2.4 <u>Nadolol</u>

<u>Plasma</u>: 2 ml plasma are shaken with 1.5 g sodium chloride and 1 ml 5N NaOH for 5 min. Then 5 ml of diethyl ether are added and the tubes are shaken again for 10 min, after which the layers are separated by centrifugation. The organic layer is transferred completely into another tube and evaporated to dryness at 40°C under nitrogen. The residues are dissolved in 50 μl methanol and 40 μl of the solution are spotted on a silica gel plate using a Linomat III (width of the strip 6 mm). Together with the extracts of twelve samples with unknown nadolol content, the extracts of four blank plasma samples (2 ml) spiked with 60, 100, 200 and 600 ng are spotted to establish the calibration graph.

The plates are developed in a saturated glass tank containing the solvent system chloroform–methanol–glacial acetic acid (75:20:5) for 8 cm. After air drying the plates are dipped into a solution of 4% paraffin in cyclohexane. After another drying period of at least 20 min, fluorescence is read (excitation wave length: 265 nm; emission wave length: 313 nm). The concentrations of nadolol in plasma are determined by a calibration graph by plotting the peak heights versus the nadolol contents in the spiked samples.

Urine: 0.5 ml of 1N HCl is added to 0.2 ml of urine. The acidic solution is pre-extracted by shaking with 5 ml diethyl ether for 5 min. The layers are separated by centrifuging and the organic layer is discarded. 1 g of NaCl and 1 ml of 5N NaOH are added to the aqueous phase, then nadolol extraction, evaporation of the solvent and spotting the TLC plates are performed as described. Again four samples of spiked urine (60, 200, 400 and 600 ng per 0.2 ml) are processed simultaneously with twelve samples with unknown nadolol content. Urine extracts are chromatographed in the solvent system chloroform-methanol-glacial acetic acid (60:35:10). After drying and dipping into the paraffin solution the plates are scanned as described for plasma.

The native fluorescence of nadolol is increased only two-fold by spraying the plate with a mixture of citric acid in water-ethylene glycol but about five-fold by covering the plate with paraffin. Then the detection limit of nadolol is about 5 ng/spot. The calibration curve is linear up to 1400 ng/spot. Use of either peak height or peak area for the calibration curve proved to be of the same quality. Peak height is chosen for analysis as it is obtained more easily. Extraction of nadolol from urine and plasma is performed by a procedure similar to that of the fluorimetric method based upon the oxidation of nadolol and coupling of the resulting aldehyde with o-phenylenediamine (ref. 54) except that plasma samples are also extracted by ether. Thus evaporation of butyl acetate, which has a rather high boiling point leading to a prolonged evaporation time, is avoided. The extraction of nadolol from plasma and urine is incomplete (63 and 77% respectively). Therefore, spiked samples have to be carried through the analysis together with samples of unknown drug content to avoid major fluctuations in the results due to day to day variation. Chromatography of nadolol extracted from plasma

in the solvent system described by Dreyfuss (ref. 28) leads to chromatograms free from interfering peaks. Chromatography of urine extracts following the same extraction schedule gives an additional peak interfering with nadolol. This can be avoided by increasing the amounts of methanol and acetic acid in the chromatographic system together with a pre-extraction of the acidified urine specimens with ether. The R_f values of nadolol chromatographed in the solvent system described for the determination in plasma and urine are 0.26 and 0.47. Reproducibility of the method is sufficient for the determination of nadolol in biological fluids. The <u>variation coefficient</u> is usually less than 4% for plasma samples and about 7% for urine (ref. 29). The method served for the determination of nadolol in plasma and urine of healthy volunteers dosed 60 and 120 mg orally (ref. 55).

5.3.2.5 <u>Penbutolol</u>

The first fluorodensitometric method for the determination of penbutolol was described in 1979 by Müller and coworkers. In this procedure quinidine serves as internal standard. Due to major differences in the fluorescence behaviour of penbutolol and quinidine, however, each TLC plate has to be scanned twice. The method was used for the determination of penbutolol levels in serum from healthy volunteers following the oral administration of 20 and 40 mg as single doses and during repeated ingestion. The pharmacokinetic behaviour of penbutolol and its effect on heart rate was evaluated in detail. However, informations on the R_f values of penbutolol and the internal standard as well as on the precision of the assay procedure are not given. The limit of penbutolol detection is 10 ng/ml (ref. 37).

In 1985 Gottschalk and Sistovaris described another procedure for the determination of penbutolol serum levels based upon a less extensive sample clean up. This method was used for the evaluation of the influence of furosemide on penbutolol serum protein binding (ref. 20).

<u>Method I</u>: 50 ng quinidine and 0.5 ml of 0.1N NaOH are added to 2 ml of <u>serum</u>. The serum is shaken for 10 min with 8 ml chloroform. After centrifugation, the aqueous layer is discarded and penbutolol re-extracted into 2 ml of 0.2N H_2SO_4 by shaking for

10 min. After aspiration of the aqueous phase the organic layer is transferred to another tube. Following the addition of two drops of 2% methanolic HCl solution the chloroform is evaporated to dryness at 40°C under nitrogen. The residue is dissolved in 50 µl chloroform containing 0.1% diethylamine. The solution is spotted quantitatively onto a silica gel 60 plate using a glass microcapillary. Together with the samples with unknown penbutolol content four serum standards containing 80, 160, 320 and 640 ng/ml are processed. Their extracts are spotted onto the same TLC plate for the construction of the calibration graph. The chromatogram is developed for 8 cm in dioxane-concentrated ammonium hydroxide solution (99:1). After removal from the chamber, the plates are dried by heating on a hot plate at 60°C for 5 min. Prior to fluorescence reading, the plates are dipped into a 10% solution of paraffin in petroleum ether and dried again at room temperature. The respective excitation and emission wave lengths are 274 and 300 nm for penbutolol and 300 and 450 nm for quinidine. Drug concentrations are calculated from the calibration graph constructed by plotting peak height ratios of penbutolol/quinidine against the penbutolol concentration.

Method II: After the addition of 0.5 ml 1M carbonate buffer pH 10 to 0.5 ml serum, penbutolol is extracted with 5 ml diethyl ether for 20 sec using a vortex mixer. The phases are separated by centrifugation. 4 ml of the organic layer are transferred to another tube and evaporated to dryness at 40°C under a stream of nitrogen. The residue is dissolved in 100 µl of methanol. 70 µl are applied onto a HPTLC plate using an Autospotter as a series of consecutive small droplets of approx. 100 nl in volume. The chromatogram is developed in a twin-trough HPTLC developing chamber (Camag, Muttenz) containing 10 ml of the solvent system dichloromethane-methanol-glacial acetic acid (9:1:0.1). The chromatogram is developed for 7 min in the dark without prior saturation. After drying, the plate is scanned (excitation wave length: 270 nm; emission wave length: 313 nm). Calibration functions (refs. 56, 57) are determined from the peak height of the standards:

$$C = \frac{C_{max} \times E_{rel} \times K_m}{1 - E_{rel} + K_m}$$

with C_{max} = maximum calibration standard, E_{rel} = peak height/maximum peak height and K_m = parameter of nonlinearity. The latter obtained from a Hofstee plot following normalization of peak height and concentrations.

The detection limit is 60 ng/ml serum and the <u>assay precision</u> 30 ng/ml + 2.5% of amount (ref. 20).

The latter method was developed for penbutolol determination in serum samples spiked with this drug and furosemide for in vitro experiments. Therefore, the procedure was tested for interference of furosemide but not for the possible interference of 4-hydroxypenbutolol. This substance is the most important one of several active penbutolol metabolites (refs. 37, 58). Penbutolol and 4-hydroxypenbutolol can be determined simultaneously using HPLC-fluorimetry (refs. 59, 60). The metabolite is extractable from plasma with ether and emits fluorescence light under the experimental conditions described by Gottschalk and Sistovaris for the determination of the parent drug (ref. 60). Therefore method II should not be used for the determination of penbutolol in patient plasma without the prior evaluation of the selectivity of the method. Filtering off the emitted fluorescence light of penbutolol at 300 nm, however, as described by Müller and coworkers (ref. 37), will reduce possible interference considerably. In the mobile phase used by Miner and coworkers 4-hydroxypenbutolol emits only minor amounts of fluorescence light at this wave length (ref. 60).

5.3.2.6 Pindolol

Pindolol can also be determined by measuring the native fluorescence of this drug on TLC plates. Before the extraction of pindolol from plasma samples nadolol is added as an internal standard. With urine samples, however, an internal standard is not added (ref. 30). As described above, the detection limit of the drug extracted from plasma could not be improved by spraying the plate with o-phthaldialdehyde solution. The reaction with o-phthaldialdehyde was used for the fluorimetric determination of pindolol with fluorescence reading in cuvettes (ref. 44). On

thin-layer chromatograms of the extracts from biological mate-
rial with low pindolol concentrations, the fluorescence of par-
tially interfering peaks increases to the same extent as pindo-
lol fluorescence intensity. Thus, in contrast to the situation
with pure substance, there is no benefit to be derived from this
treatment (ref. 30).

Extraction from <u>plasma</u> samples: 200 ng of nadolol, 0.5 g NaCl
and 1 ml 5N NaOH are added to 2 ml plasma and the mixture is
shaken with 5 ml of methylene chloride-diethyl ether (20:80) for
15 min. After centrifugation, 4 ml of the organic layer are
transferred to another tube and evaporated to dryness at 50°C
under an atmosphere of nitrogen.

Extraction form <u>urine</u> samples: 50 µl of 1N NaOH, 0.5 g NaCl
and 6 ml of n-butyl acetate are added to 0.5 ml of urine. The
mixture is shaken for 20 min and thereafter centrifuged. 5 ml of
the butyl acetate layer are removed and evaporated to dryness in
a vacuum centrifuge at 30°C.

TLC, fluorescence enhancement and densitometry: The extracts
from plasma (urine) are dissolved in 50 µl methanol (butyl ace-
tate) and 40 (35) µl are spotted onto a silica gel 60 plate in a
5-mm strip. To establish the calibration graph, the extracts of
three plasma or urine samples containing 50, 100 and 200 ng/ml
plasma or 200, 600 and 1000 ng/ml urine are spotted on each
plate. The chromatogram is developed for 12 cm in an unlined
glass tank containing chloroform-methanol-acetic acid (75:20:5).
After air drying the plate is dipped into a 4% solution of li-
quid paraffin in cyclohexane. After another drying period the
plate is scanned (excitation wave length: 265 nm; emission wave
length: 313 nm). Peak areas serve for the construction of the
calibration graphs. If an internal standard is used, the peak
area ratio is calculated.

The R_f values of pindolol and nadolol are 0.45 and 0.24,
respectively. Chromatograms of plasma extracts are free from in-
terfering substances whereas urine constituents influence the
nadolol peak. Therefore this drug cannot be used as an internal
standard for the evaluation of pindolol levels in urine. The
pindolol metabolite does not interfere with the analytical pro-
cedure. Neither do many antirheumatic/analgesic drugs, the β-ad-
renoceptor antagonists propranolol, metoprolol, atenolol and
quinidine, all of which have been tested for interference. How-

ever, hydrochlorothiazide and triamterene may interfere, if they are present in high concentrations.

The plates have to be scanned rapidly after the fluorescence enhancement as pindolol decomposes on TLC plates (Table 5.3.1). The detection limit of pindolol is 0.5-1 ng/spot. The detection limit in plasma is 2 ng/ml, in urine 40 ng/ml. Using HPTLC plates, the detection limit in plasma is 1 ng/ml. Mean recovery rates are 93% for plasma and 71% for urine. The calibration graphs are linear up to 350 and 4000 ng/ml, respectively. The coefficient of variation is 3.7% at a plasma concentration of 50 ng/ml and 5.5% at 10 ng/ml. For urine it amounts to 5.5% at 400 ng/ml. The method proved to be suitable for the determination of pindolol plasma levels for 12-24 h following a chronic administration of 10 mg (ref. 30).

TABLE 5.3.1 Changes in fluorescence intensity of pindolol (1µg/spot) with time. (A) During storage on the TLC plates in the dark and under vacuum conditions; (B) while exposed to light and air (from ref. 30; with permission of the publisher).

Time	Fluorescence intensity (%)		Intensity of violet colour of the decomposition product*	
(h)	A	B	A	B
0.0	100	100	−	−
0.5	100	69	−	+
1.0	100	60	−	+
1.5	100	52	−	++
2.0	100	40	−	+++
5.0	90	28	(+)	++++

*−, No colour; (+), weakly visible; +, visible (the number of +-signs indicates the intensity of the colour).

5.3.2.7 Propranolol

Finally, propranolol and three of its metabolites can be determined in biological material by fluorodensitometry (refs. 24, 38). The latter method has been modified to allow the use of an automatic spotting device. Moreover, fluorescence enhancement by spraying with water-ethylene glycol-citric acid has been replaced by dipping into a paraffin containing solution (refs. 26, 36). This version is reported here (method I). In addition, the methods developed by Garceau and coworkers are described, since these procedures allow not only the determination of the parent drug but also of three propranolol metabolites (method II; ref.

24). Two further methods also use TLC for the separation of pro-
pranolol from co-extracted impurities but use UV absorbance for
the quantitation (refs. 41, 42). However, their limits of detec-
tion exceed that of the fluorimetric methods. As these methods
offer no advantages over the fluorimetric procedures they are
not reported here.

Method I: 0.5 g NaCl, 0.5 ml 1N NaOH and 2.5 ml pentanol-hep-
tane (3:97) are added to 1 ml plasma or urine and the mixture is
shaken for 15 min. After centrifugation, 2 ml of the organic
layer are transferred to another tube and evaporated to dryness
at 50°C under nitrogen. The residue is dissolved in 50 µl of
pentanol-heptane and 40 µl are spotted onto a silica gel 60
plate by means of a Linomat III. Three standards (spiked with
100 ng propranolol HCl/ml) are proceeded with every 12 plasma
samples containing unknown propranolol concentrations. The chro-
matogram is developed in a saturated glass tank containing the
solvent system chloroform-methanol (97:3) in an atmosphere of
ammonia. After drying, the plate is dipped into a solution of 4%
paraffin in cyclohexane. After a further drying period, fluo-
rescence is read (excitation wave length: 265 nm; emission wave
length: 365 nm). Concentrations are calculated from the areas
under the peaks as compared to the standards.

The R$_f$ value of propranolol is 0.19. Recovery rates of pro-
pranolol from plasma and urine amount to 70 and 77% respecti-
vely. The coefficients of variation are 3.0-6.7% (50-500 ng/ml).
The limit of detection is about 3 ng/ml. The method served, for
example, for the determination of propranolol in plasma and
urine following the oral administration of 60-80 mg propranolol
HCl alone and in combination with hydrochlorothiazide/triam-
terene or bendroflumethiazide/triamterene. No interferences due
to these substances were observed (refs. 61, 62). Furthermore, a
good agreement between propranolol plasma levels determined by
this method and by a radioreceptor assay was obtained (r=0.93,
slope 1.01; ref. 26).

Method II: Determination of free propranolol: 1 ml 1N NaOH
and 40 ml of ether are added to 5 ml plasma or urine and the
mixture is shaken for 15 min. After centrifugation the ether is
transferred and dried with 12 g sodium sulfate. 25 ml of the
dried ether phase are removed and evaporated at 45°C under ni-
trogen, the inside of the tube is washed with 1 ml of ethanol

and the solution is evaporated to dryness. The residue is dissolved in 100 µl of ethanol and 25 µl are spotted onto a silica gel 60 plate. Standard solutions of propranolol are spotted on the same plate. The chromatogram is developed for 12 cm in a saturated tank containing methanol (100 ml) and concentrated ammonium hydroxide (0.4 ml). The air dried plate is sprayed with propylene glycol-water (1:1), then the plate is scanned (excitation wave length: 290 nm; emission wave length: 365 nm). Quantitation is achieved by comparing the areas under the peaks obtained from the unknown samples to those obtained from the standards.

Determination of conjugated propranolol: Plasma (or urine) is mixed with acetate buffer pH 4.7 (0.1M) and enzyme solution containing β-glucuronidase and sulfatase. The mixture is incubated at 37°C for 1.5 (17) h, after which the solution is made alkaline by adding 1N NaOH and propranolol is extracted with ether. The extracts are chromatographed on silica gel plates in a saturated tank containing benzene-isopropyl alcohol-dimethylformamide-acetic acid (70:10:10:10). Spraying the plate with propylene glycol-water and fluorescence reading are performed as described above. The procedure quantitates total propranolol (free plus conjugated drug). Conjugated propranolol is obtained by subtracting free from total drug.

Determination of naphthoxylactic acid: Incubation of plasma and urine samples with β-glucuronidase/sulfatase as well as the extraction of the alkaline samples with ether is performed as described for conjugated propranolol. The ether is discarded. The aqueous phase is acidified with HCl and extracted with ether. The further procedure corresponds to that of conjugated propranolol. The procedure determines the concentrations of total naphthoxylactic acid. Free acid can also be evaluated, omitting the enzyme hydrolysis.

Determination of 4-hydroxypropranolol: Extraction and evaporation steps are carried out in polypropylene tubes. Plasma (urine) is mixed with acetate buffer and glusulase solution and incubated at 37°C for 30 (20) min. Thereafter pH 10 buffer is added and the mixture is extracted with refluxed ether.

Chromatographic separation is performed on microcrystalline cellulose plates in the solvent system ethyl acetate-acetone-water (40:45:15). After drying fluorescence is read (excitation:

300 nm, emission: 420-440 nm). The procedure measures total 4-hydroxypropranolol. Free drug can also be determined omitting the enzyme hydrolysis.

In the chromatographic system used for the determination of free propranolol the drug has a R_f value of 0.34. Thus propranolol is well separated from the basic metabolite desisopropylpropranolol (R_f: 0.15) and the neutral one 1-(α-naphthoxy)-2,3-propanediol (R_f: 0.84). The limit of quantitation is 2 ng/ml for both plasma and urine; instrument response is linear from 0-100 ng/spot. <u>Recovery rates</u> of propranolol and its metabolites are summarized in Table 5.3.2. They are close to 100%. However, spraying the plate with propylene glycol is a critical step in the procedure as discussed above. Overspraying results in an erratic baseline, while underspraying results in a loss of sensitivity. This can be avoided with fluorescence enhancement by covering the plate with paraffin which leads to a comparable detection limit. This technique may possibly also be suitable for the fluorescence enhancement of 4-hydroxypropranolol and naphthoxylacetic acid.

TABLE 5.3.2 Recoveries of propranolol, naphthoxylactic acid and 4-hydroxypropranolol from plasma and urine under the conditions of the fluorodensitometric method described by Garceau and co-workers (from ref. 24).

Substance	Plasma				Urine			
	Amount spiked (ng/ml)	Mean recovery (%)	SD (%)	n	Amount spiked (µg/ml)	Mean recovery (%)	SD (%)	n
Propranolol	5-50	95.1	6.5	24	.001-1	92.4	8.7	12
Propranolol*	25-500	97.7	6.3	15	.10-.75	101.9	5.2	12
Naphthoxy-lactic acid	250-1000	94.4	6.9	12	2.5-10.0	96.5	7.4	8
4-Hydroxypr.	10-500	96.4	7.8	18	.10-1.0	83.6	5.7	8
4-Hydroxypr.*	50-1000	64.8	6.1	15	5-25	73.5	5.9	8

*Spiked samples were submitted to the procedure to assay conjugated substance.

In the solvent system used for the determination of conjugated propranolol, the parent drug and its metabolites are also well separated. The respective R_f values read propranolol, 0.20; desisopropylpropranolol, 0.07 and 1-(α-naphthoxy)-2,3-propanediol, 0.67. The presence of acidic metabolites is excluded by the high extraction pH. The R_f values read 4-hydroxypropranolol, 0.06; p-hydroxy-1(α-naphthoxy)-2,3-propane-

diol, 0.43; naphthoxylactic acid, 0.54; dihydroxynaphthalene, 0.61; naphtoxyacetic acid, 0.71; and α-naphthol, 0.74. Propranolol is stable under the conditions used for the enzyme hydrolysis leading to complete hydrolysis of propranolol conjugates in patient plasma and urine samples. The limits of quantitation are 15 ng/ml of plasma and 50 ng/ml of urine.

In the chromatograms of the extracts of acidified plasma and urine samples, naphthoxylactic acid can be identified, the metabolite being free from interference due to other metabolites and substances co-extracted from the biological material. The areas under the peaks are linearily related to the drug amount/spot in the range of 0-250 ng. The limits of determination are 25 and 125 ng/ml respectively.

4-hydroxypropranolol shows also strong fluorescence. The emission maximum is between 400 and 450 nm. In the respective chromatographic system the R_f value of this metabolite is 0.57. It is well separated from co-extracted plasma and urine components although baseline separation is not obtained. Propranolol and desisopropylpropranolol spots partially overlap with the 4-hydroxypropranolol spot (R_f values: 0.59 and 0.53). However, due to their maximum fluorescence emission at 365 nm, they are filtered off and thus do not interfere with the 4-hydroxypropranolol assay. The limits of quantitation for free 4-hydroxypropranolol are 10 ng/ml plasma and 100 ng/ml urine and for the conjugates 50 and 2000 ng/ml. The recovery of free 4-hydroxypropranolol from plasma and urine is nearly complete. Due to the instability of this metabolite during the enzyme hydrolysis, the recovery of total 4-hydroxypropranolol is lower (Table 5.3.2). Degradation during the hydrolysis procedure is kept to a minimum by high enzyme concentrations and short incubation at low temperature. The sensitivity of this metabolite to metal ions requires the use of tubes containing ethylenediaminetetraacetic acid for the collection of blood and the use of polypropylene tubes for the evaporation of the ether extracts. Instability on TLC plates is excluded by chromatography on microcrystalline cellulose plates. The degradation favoured by peroxides can be circumvented by using refluxed ether. In plasma samples kept at -20°C 4-hydroxypropranolol is stable for one month.

5.3.3 DETERMINATION OF CARTEOLOL AND ITS METABOLITES BY UV AB-
    SORBANCE

In contrast to many other β-adrenoceptor antagonists carteo-
lol does not show an intrinsic fluorescence. Moreover formation
of fluorescing derivatives with EDTN, DANS-Cl and BOP-Cl failed
to work in our laboratory. For the dansylation at least, this
observation can now be explained by the results of the experi-
ments of Schulz and Zapka which demonstrated that β-adrenoceptor
blockers containing a tert. butyl moiety do not react with
DANS-Cl. Therefore an effort was made to develop a method for
the determination of carteolol and its metabolites based upon
the UV absorbance at 254 nm (refs. 26, 27).

Carteolol in plasma samples: 10 µg nadolol (internal stan-
dard), 0.5 g NaCl, 1 ml 1N NaOH and 5 ml diethyl ether are added
to 2 ml plasma. After shaking for 7 min the layers are separated
by centrifugation. 4 ml of ether are transferred to another tube
and evaporated to dryness at 50°C under nitrogen. The residue is
dissolved in 50 µl of methanol. 40 µl are applied onto a silica
gel 60 plate as a 6 mm strip (Linomat III). The chromatogram is
developed for 10 cm in a saturated glass tank containing chloro-
form-methanol-glacial acetic acid (75:20:4.5). After drying, UV
absorbance of carteolol and nadolol is read at 254 nm. Carteolol
concentrations are calculated from the peak height ratios
(carteolol/nadolol) of the unknown samples as compared to those
of four standards which are run on each plate. These contain 25,
50, 100 and 150 ng carteolol/ml plasma.

Carteolol in urine samples: 50 µl of urine is added to 0.5 ml
1N NaOH and shaken for 10 min with 5 ml ether. After centrifuga-
tion the organic phase is transferred completely to another tube
and evaporated to dryness. The residue is dissolved in 50 µl me-
thanol and 20 µl are applied to a TLC plate as described. Toge-
ther with 12 urine samples with unknown carteolol content, 3
standards are processed which contain 10 µg/ml. The chromatogram
is developed for 10 cm in a saturated glass tank containing
chloroform-methanol-glacial acetic acid (75:20:3). Thereafter
the plate is scanned as described for plasma. Carteolol concen-
trations of the unknown samples are calculated from the peak
heights as compared to the standards.

The carteolol-glucuronide concentration in urine can be de-
termined after hydrolysis with glusulase. Furthermore 8-hydroxy-
carteolol can be quantified in urine, if the degradation of this
metabolite is avoided by adding NaHS$_2$O$_5$ at the end of each
collection period. The metabolite is extracted with chloroform.
Chromatography is performed on silica gel plates in the solvent
system   chloroform-methanol-n-heptane-glacial   acetic   acid
(65:20:10:5).

In the solvent system used for the chromatography of plasma
extracts the R$_f$ values of carteolol and nadolol are 0.34 and
0.18. The limit of carteolol detection is about 7 ng/ml. This is
the major drawback of the procedure as it excludes carteolol de-
termination following the administration of therapeutic doses
(5-10 mg). The limit of carteolol detection using densitometry,
however, is comparable to that of the fluorimetric method de-
scribed by Morita (irradiation of a solution in hydrochloric
acid with a tungsten lamp; ref. 63) and a HPLC procedure (ref.
64). The detection limit is much lower using a radioimmunoassay
(ref. 65) and the radioreceptor assay (ref. 26).
The recovery of carteolol from plasma is 56% and the coefficient
of variation amounts to 8-10% at 25-100 ng/ml. The densitometric
method is specific for the parent drug. Determination of carteo-
lol concentration by this method as well as by the radioreceptor
assay led to identical results, if spiked samples were analysed.
However, carteolol concentrations in patient plasma were always
higher using the radioreceptor assay as compared to the densito-
metric method. This results from the interference of 8-hydroxy-
carteolol which occurs when the former procedure is used (ref.
26).

From urine samples 79% of carteolol is recovered. The parent
drug is well separated from 8-hydroxycarteolol (R$_f$ values: car-
teolol 0.30; 8-hydroxycarteolol 0.22). The coefficient of varia-
tion is 3.4 to 9.0% at concentrations ranging from 2 to 50
µg/ml. 8-hydroxycarteolol also shows strong UV absorbance. In
the chromatographic system described, it is well separated from
urine components and from carteolol (R$_f$ values: carteolol 0.35;
8-hydroxycarteolol 0.23). Recovery from urine shows a rather
high day to day variation (49-69%), which is compensated by
using urine standards. The coefficient of variation is 4.8 to
7.0% at concentrations of 1 to 6 µg/ml. The method served for

the evaluation of the influence of renal failure on carteolol pharmacokinetics. The patients received single oral doses of 30 mg carteolol HCl (ref. 27).

5.3.4 DETERMINATION OF β-ADRENOCEPTOR ANTAGONISTS BY THE FORMA-
TION OF EDTN-DERIVATIVES

EDTN (Fig. 5.3.1) was first used for the assay of corticoste-roids (ref. 66). Rapid methods for the determination of two β-adrenoceptor blockers, tolamolol and oxprenolol, were published, too (refs. 31, 32). Both drugs, as well as 1-(2-(2-carbamoyl-4-methylphenoxy)-ethylamino)-3-(2-methylphenoxy)-propan-2-ol   (a tolamolol derivative) and metoprolol which serve as internal standards, rapidly form blue-fluorescing derivatives with EDTN (Fig. 5.3.4). These are stable for several days on paraffin-co-vered TLC plates. After the reaction, the solution can be ap-plied directly onto the plate. Another extraction step, which is required after dansylation (e.g. ref. 12) is not necessary. The minimum detectable levels of both tolamolol and oxprenolol are rather low (2 and 5 ng/ml).

5.3.4.1 Tolamolol

100 ng of the internal standard (see above) is added to 1 ml plasma or serum. The drugs are extracted by shaking for 10 min with 4 ml ethyl acetate. After centrifugation, the organic layer is transferred into another tube and evaporated to dryness at 40°C under nitrogen. The residue is dissolved in 0.5 ml methanol and extracted with 1 ml heptane by shaking for 20 sec. After centrifugation the heptane is discarded and the methanol evapo-rated as described. The residue is dissolved in 50 µl of EDTN solution in dry ethyl acetate (0.2 mg/ml). The reaction is allo-wed to proceed for 10 min at 40°C. The ethyl acetate solution is applied quantitatively onto a silica gel 60 plate (spot size be-low 4 mm). The chromatogram is developed in a saturated tank containing hexane-isopropanol (75:25) for 15 cm. Then the plate is dried and the fluorescent spots stabilized by spraying with liquid paraffin-diethyl ether (1:2). Fluorescence of the EDTN-derivatives of tolamolol and the internal standard is excited
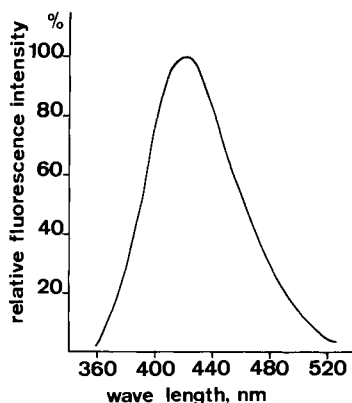
Fig. 5.3.4. Emission spectrum of oxprenolol derivatised with EDTN (excitation wave length 313-nm; from ref. 32 with permission of the publisher)

with UVB-light, the emission wave length is 430 nm. Plasma standards, spiked with 20-200 ng tolamolol free base/ml serve for the construction of the calibration curve.

The R_f values of tolamolol and the internal standard are 0.23 and 0.35 respectively. The assay is specific for tolamolol. The EDTN derivative of a hydroxylated metabolite which is extractable with ethyl acetate has a R_f value of 0.14. The limit of detection is 2 ng/ml. The <u>recovery</u> from plasma amounts to 98%, the <u>coefficient of variation</u> is 3.6% at 100 ng/ml (ref. 31).

5.3.4.2 Oxprenolol

100 ng metoprolol tartrate, 0.5 ml 1N NaOH and 0.25 g NaCl are added to 1 ml <u>plasma</u>. The mixture is shaken for 15 min with 5 ml dichloromethane-diethyl ether (1:4). After centrifugation, the organic phase is transferred into another tube and evaporated to dryness at 50°C under nitrogen. 40 µl EDTN solution (0.2 mg/ml ethyl acetate) are added to the residue and the stoppered tube is heated to 50°C for 15 min. After cooling to room temperature the entire ethyl acetate volume is applied onto a TLC plate as described for tolamolol. Together with a series of unknown samples, three standards are spotted per plate. The

standards are carried through the analysis with the other samples. The chromatogram is developed in an unlined glass tank containing chloroform-ethyl acetate (95:5) for 15 cm. After drying the plate is dipped into a solution of 2% paraffin in cyclohexane. After another drying period of at least 15 min the plates are scanned (excitation: 365 nm; emission: 436 nm). The peak height ratios oxprenolol/metoprolol are calculated.

The reaction of oxprenolol and metoprolol with EDTN remains constant under widely varying conditions (reaction time 10-60 min, temperature 35-70°C). Oxprenolol and metoprolol are well separated from the plasma constituents. The R_f values are 0.62 and 0.41 respectively. The minimum detectable level of oxprenolol is 5 ng/ml. Standard curves are linear from 10-1000 ng oxprenolol HCl and pass through the origin. Recovery of oxprenolol from plasma is 82%. At therapeutic plasma concentrations the coefficient of variation is 4.1%. Atenolol, propranolol, digitoxin, quinidine, guanethidine, hydralazine, triamterene and diazepam do not interfere with the assay procedure.

5.3.5 DETECTION OF β-ADRENOCEPTOR ANTAGONISTS BY TLC – APPLICATION TO COMPLIANCE CHECK AND ANALYTICAL TOXICOLOGY

As described, qualitative methods are used particularly for the evaluation of patient compliance and in forensic medicine. This requires a low limit of drug detection. In analytical toxicology, methods are looked for which allow rapidly the exclusion of drugs which are not present in the sample. Only a few "candidate drugs" should be left.

For the detection of non-compliance of patients on antihypertensive therapy, Jack and coworkers developed a TLC procedure for β-adrenoceptor antagonists as well as metabolites of hydralazine and dihydralazine (ref. 1, 2).

Extraction: Except from sotalol and labetalol 4 ml urine are made alkaline with 50 μl 7N NaOH. For the extraction of labetalol, 2 ml 1M ammonia-HCl buffer pH 8.5 is substituted for the NaOH. The mixture is extracted with 4 ml diethyl ether-dichloromethane (4:1) for 5 sec. After separation of the phases the organic layer is transferred into another tube and evaporated under nitrogen. For the extraction of sotalol, 4 ml of 0.1M carbonate-bicarbonate buffer pH 10.0 and 0.1 ml charcoal slurry

(suspension of 0.5 g activated charcoal in 100 ml carbonate-bi-carbonate buffer) is added. The tube is mixed for 5 min and cen-trifuged at 300 g for another 5 min. The supernatant is discar-ded and the residue washed with 10 ml distilled water by mixing for 2 min. It is then centrifuged and the water is discarded. 2 ml methanol are added to the residue and mixed for 10 sec on a vortex mixer. After standing for 5 min the sample is centrifuged again. The methanol is decanted into another tube and evaporated at 50°C under nitrogen.

Chromatography and detection: The residues are dissolved in 1-2 drops of methanol and spotted onto a silica gel plate toge-ther with appropriate standards. The chromatogram is developed in the solvent system ethyl acetate-methanol-ammonia (40:5:5) for 8 cm. After drying, the plate is examined under UV light at 254 nm. A large beaker is then placed below the plate and the appropriate visualizing reagent (concentrated sulfuric acid, 38% formaldehyde in concentrated sulfuric acid (1:10) and a satura-ted solution of ammonium metavanadate in concentrated sulfuric acid) is poured down the plate. The R_f values of the investiga-ted drugs as well as their characteristic colours and the mini-mum detectable level are summarized in Table 5.3.3. Both acebu-tolol and its metabolite diacetolol show up as yellow spots. Metoprolol is not detected itself, but the renally excreted 1-hydroxy-2-methoxyethyl metabolite gives an intense purple colour with concentrated sulfuric acid. The methods are sufficient for the detection of the drugs (and/or their metabolites) in urine after the application of therapeutic doses. This also holds true for sotalol despite the rather low sensitivity. Sotalol is ex-creted essentially unchanged by the kidney and its therapeutic doses are rather high. If the presence of hydralazine and dihy-dralazine metabolites is looked for, another TLC separation car-ried out before the reported one is necessary (refs. 1, 2).

Urine concentrations of acebutolol and diacetolol are suffi-ciently high to allow direct spotting of urine onto the plate. Therefore the extraction step can be omitted (ref. 1). Using another reagent, it is also possible to detect pindolol in urine samples without prior extraction. The plate is developed in the solvent system chloroform-methanol (95:5; atmosphere of ammo-nia). After drying of the plate and complete evaporation of the ammonia pindolol is detected by applying about 50 µl of p-dime-

TABLE 5.3..3 Rf values of β-adrenoceptor blockers (plates: TLC silica gel; solvent: ethyl acetate-methanol-ammonia (40:5:5)), characteristic colours and minimum detectability in urine (from refs. 1, 2). Reagents: I, concentrated sulfuric acid; II, 38% formaldehyde in concentrated sulfuric acid (1:10); III, saturated solution of ammonium metavanadate in concentrated sulfuric acid.

Compound	Rf	UV254	Colour with:			Minim. detect. (mg/l)
			Reagent I	Reagent II	Reagent III	
Acebutolol	0.73	yellow	yellow -blue	faint yellow		0.05+
Diacetolol	0.60	yellow	yellow -blue	faint yellow		0.05+
Alprenolol	0.90	-		red brown	red brown	0.01
Atenolol	0.54	-		-	brown	0.5
Labetalol	0.76	pale blue		-	green	0.5
Metoprol.*	0.65	-	purple	yellow		0.5
Nadolol	0.50	-	-	orange-red		1.0
Oxprenol.	0.81		faint purp.	purple		0.5
Pindolol	0.84	-		yellow	brown	1.0
Propranol.	0.81	-	faint brown	green		0.5
Sotalol	0.70	-		-	brown	50

+Detected by fluorescence at 254 nm
*Metabolite

thylaminobenzaldehyde reagent (prepared by dissolving 1 g of p-dimethylaminobenzaldehyde in 100 ml ethanol and adding 10 ml concentrated HCl) to the area of the Rf value (0.33). After drying, a blue colour develops, if pindolol concentration exceeds 3 ng/spot. Thus pindolol is detectable in urine for at least 8 h after the ingestion of a therapeutic dose (ref. 30).

Screening procedures for β-adrenoceptor blocking drugs in urine based upon TLC separation and consecutive colour reactions also have been reported by Michaud and Jones (ref. 3) and Bonicamp and Pryor (ref. 4). Both methods use TOXI-TUBES A^R (Analytical Systems) for the extraction of the drugs from urine (at pH 9.0). The organic solvents used for the extraction of the drugs from the tube are concentrated and transferred to silica gel plates (refs. 3, 4). After chromatography the drugs are detected by dipping the plates into several reagents sequentially and observation of the resulting colour development. The limits of detection correspond to those reported by Jack and coworkers (refs. 1, 2). Due to the sequential colour reactions (refs. 3, 4) the danger of false-positive results should be less than that seen with the former method (refs. 1, 2).

A fluorimetric method for the identification of β-adrenoceptor blocking agents containing an isopropyl residue by two-dimensional TLC has been described by Schulz and Zapka. The drugs are identified by means of their DANS derivatives which excludes the detection of those β-adrenoceptor antagonists whose secondary amino group bears a tertiary butyl residue due to steric hinderance. The reaction takes place in aqueous acetone at pH 9.8 at 55°C over 20 min in the darkness. After cooling to room temperature, the yellow fluorescing DANS derivatives are extracted with diethyl ether. The ether volume is reduced by evaporation and a few microliters are spotted onto a silica gel 60 plate (20x20 cm). The chromatogram is developed first in benzene-cyclohexane-methanol (75:10:15, atmosphere of ammonia) and then in cyclohexane-n-butyl acetate (75:25) as the mobile phase at right angle to the first separation. However, a two-dimensional separation is only necessary, if oxprenolol is to be separated from toliprolol and/or propranolol from alprenolol. The limit of visual detection under a UV lamp (366 nm) is 2-100 ng/spot (ref. 12).

No extracts of biological material have as yet been submitted to this procedure. Such material, however, often contains impurities which will react with DANS-Cl. Thus a lot of spots will be found which are not derivatives of β-adrenoceptor antagonists, and which may complicate the correct identification of the drugs. Due to the low selectivity of DANS-Cl, the method bears also a high risk of interference due to other drugs which may be present in the biological sample and may react with this fluorescence reagent.

For analytical toxicology Daldrup and coworkers present chromatographic data (TLC, HPLC, GLC) of 570 substances. However, this report deals only with pure drugs. TLC separation of β-adrenoceptor antagonists is performed for 10 cm on silica gel 60 plates with fluorescence indicator in the chromatographic system methanol-concentrated ammonium hydroxide solution (100:1.5). Most β-adrenoceptor antagonists can be visualized with Dragendorff spray reagent. Exceptions are atenolol, nadolol and sotalol (ref. 9).

Subsequently Musumarra et al. described the application of principal component analysis to TLC data of hundreds of drugs including the widely used β-adrenoceptor antagonists (refs. 10,

11). Principal component analysis is a quick and simple analytical tool for the identification of drugs (ref. 67). The number of variables characterizing a drug (first several (four) R_f values; see below) are reduced to two (Θ_1 and Θ_2 values). These scores can then be presented graphically. To identify an unknown drug, the scores are calculated from the R_f values determined under standardized condition (ref. 68) and the candidate drugs are extracted from the graph. Occasionally, the number of candidate drugs is lower using the scores as compared to the use of the R_f values itself. For the drugs studied, the Θ_1 and Θ_2 values together with the characteristic variables of the solvent system, which are essential for the calculation of the scores of unknown samples, are given in the papers (refs. 10, 11).

The drugs can be characterized by TLC (ref. 10) or HPTLC (ref. 11) using silica gel 60 plates and four different chromatographic systems. Visualization is performed again by the application of Dragendorff spray reagent. The application of four reference compounds in addition to the unknown samples makes the determination of the standardized R_f (x100) value possible (ref. 68). Using HPTLC plates leads to higher reproducibility, shorter analysis time and slightly improved sensitivity. Then 3-4 instead of 5-10 μg/spot are sufficient for detection (ref. 11).

The examination of extracts from biological material is more complicated due to interference from co-extracted biological material which alters the chromatographic data. To solve this problem, the authors suggest the preliminary chromatographic purification of the extracts in the solvent system ethyl acetate-methanol-30% ammonia (85:10:5). The silica gel areas containing the spots are scratched from the plate and extracted with methanol. The concentrated extracts are then subjected to TLC with the four chromatographic systems for the determination of the standardized R_f values (ref. 11).

222

REFERENCES

1   D.B. Jack, S. Dean and M.J. Kendall, J. Chromatogr., 187 (1980) 277–280
2   D.B. Jack, S. Dean, M.J. Kendall and S. Laugher, J. Chromatogr., 196 (1980) 189–192
3   J. Michaud and D.W. Jones, Am. Lab., 12 (1980) 104–107
4   J.M. Bonicamp and L. Pryor, J. Anal. Toxicol., 9 (1985) 180–182
5   H. Auterhoff and R. Stanke, Dtsch. Apoth. Ztg., 43 (1976) 1596–1597
6   M.E. Abdel-Hamid, M. Bedair and M.A. Korany, Pharmazie, 40 (1985) 494–495
7   M.P. Quaglio and G. Sandri Cavicchi, Il Farmaco, 30 (1975) 562–569
8   L. Slusarek and K. Florey, in K. Florey (Editor), Analytical Profiles of Drug Substances, Vol. 9, Academic Press, 1980
9   T. Daldrup, F. Susanto and P. Michalke, Fresenius Z. Anal. Chem., 308 (1981) 413–427
10 G. Musumarra, G. Scarlata, G. Romano, S. Clementi and S. Wold, J. Chromatogr. Sci., 22 (1984) 538–547
11 G. Musumarra, G. Scarlata, G. Cirma, G. Romano, S. Palazzo, S. Clementi and G. Giulietti, J. Chromatogr., 350 (1985) 151–168
12 H.G. Schulz and R. Zapka, Fresenius Z. Anal. Chem., 323 (1980) 162–167
13 N. Seiler and H. Möller, Chromatographia, 2 (1969) 273–280
14 N. Seiler and H. Möller, Chromatographia, 2 (1969) 319–324
15 N. Seiler and H. Möller, Chromatographia, 2 (1969) 470–476
16 U. Hezel, Angew. Chem., 85 (1973) 334–342
17 H.E. Geißler and E. Mutschler, Arzneim. Forsch., 28 (1978) 1964–1968
18 J.G. Touchstone and S.S. Levine, J. Liquid Chromatogr., 3 (1980) 1853–1863
19 G. Gübitz and S. Mihellyes, J. Chromatogr., 314 (1984) 462–466
20 R. Gottschalk and N. Sistovaris, Arzneim. Forsch., 35 (1985) 899–902
21 G.H. Stewart and T.D. Gierke, J. Chromatogr. Sci., 8 (1970) 129–131
22 L.E. Martin, R. Hopkins and R. Bland, Brit. J. Clin. Pharmacol., 3 (1976), Suppl. 695–710
23 J.G. Dring, A. Gorchein, A.A. Mahgoub and M. Smith, Brit. J. Clin. Pharmacol., 5 (1978) 262–265
24 Y. Garceau, I. Davis and J. Hasegawa, J. Pharm. Sci., 6 (1978) 826–831
25 F.J. Di Carlo, F.J. Leinweber, J.M. Szpiech and I.W.F. Davidson, Clin. Pharmacol. Ther., 22 (1977) 858–863
26 A. Wellstein, D. Palm, G. Wiemer, M. Schäfer-Korting and E. Mutschler, Eur. J. Clin. Pharmacol., 27 (1984) 545–553
27 G. Hasenfuß, M. Schäfer-Korting, H. Knauf, E. Mutschler and H. Just, Eur. J. Clin. Pharmacol., 29 (1985) 461–465
28 J. Dreyfuss, J.M. Shaw and J.J. Ross jr., Xenobiotica, 8 (1978) 503–508
29 M. Schäfer-Korting and E. Mutschler, J. Chromatogr., 230 (1982) 461–465
30 H. Spahn, M. Prinoth and E. Mutschler, J. Chromatogr., 342 (1985) 458–464

31 D.A. Stopher, J. Pharm. Pharmacol., 27 (1975) 133-134
32 M. Schäfer and E. Mutschler, J. Chromatogr., 164 (1979) 247-252
33 J.M. Steyn, J. Chromatogr., 120 (1976) 465-472
34 W. Möhrke, E. Mutschler, W. Mühlenberg and D. Platt, in D. Platt (Editor), Drugs and Aging, Springer, Berlin, 1986, pp. 144-151
35 M. Schäfer and E. Mutschler, J. Chromatogr., 169 (1979) 477-481
36 E. Mutschler, M. Schäfer-Korting and W. Kirch, Pharm. Ztg., 128 (1983) 124-132
37 F.O. Müller, H.K.L. Hundt, P.A. Bromley, J. Torres and O. Vanderbeke, Clin. Pharmacol. Ther., 25 (1979) 528-535
38 M. Schäfer, H.E. Geissler and E. Mutschler, J. Chromatogr., 143 (1977) 607-613
39 J.A.F. de Silva, J.C. Meyer and C.V. Puglisi, J. Pharm. Sci., 65 (1976) 1230-1233
40 S. Uchiyama and M. Uchiyama, J. Chromatogr., 153 (1978) 135-142
41 P.K. Ambler, B.N. Singh and M. Lever, Clin. Chim. Acta, 54 (1974) 373-375
42 B.W. Hadzija and A.M. Mattocks, J. Pharm. Sci., 67 (1978) 1307-1309
43 K.Y. Lee, D. Nurok, A. Zlatkis and A. Karmen, J. Chromatogr., 158 (1978) 403-410
44 W.L. Pacha, Experientia, 25 (1969) 802-803
45 L.T. Potter, J. Pharmacol. Exp. Ther., 155 (1967) 91-100
46 Doctoral Thesis G. Pflugmann, University of Francfort, 1986
47 R. Hopkins, L.E. Martin and R. Bland, Biochem. Soc. Transactions, 4 (1976) 726-729
48 W. Kirch, H. Köhler, E. Mutschler and M. Schäfer, Eur. J. Clin. Pharmacol., 19 (1981) 65-71
49 W. Kirch, M. Schäfer-Korting, E. Mutschler, E.E. Ohnhaus and W. Braun, J. Clin. Pharmacol., 23 (1983) 171-177
50 W. Kirch, H. Köhler, H. Spahn and E. Mutschler, Lancet, ii (1981) 331-332
51 W. Kirch, M. Schäfer-Korting, T. Axthelm, H. Köhler and E. Mutschler, Clin. Pharmacol. Ther., 30 (1981) 429-435
52 M. Schäfer-Korting, W. Kirch, T. Axthelm, H. Köhler and E. Mutschler, Clin. Pharmacol. Ther., 33 (1983) 283-288
53 H.R. Ochs, D.J. Greenblatt, R.M. Arendt, M. Schäfer-Korting and E. Mutschler, Arzneim. Forsch., 35 (1985) 1580-1582
54 E. Ivashkiv, J. Pharm. Sci., 66 (1977) 1168-1172
55 M. Schäfer-Korting, N. Bach, H. Knauf and E. Mutschler, Eur. J. Clin. Pharmacol., 26 (1984) 125-127
56 G. Kufner and H. Schlegel, J. Chromatogr., 169 (1979) 141-152
57 N. Sistovaris, GIT Fachz. Lab. (1983; Suppl. 3) 17-18
58 J.F. Guidicelli, C. Richer, M. Chauvin, N. Idrissi and A. Berdeaux, Brit. J. Clin. Pharmacol., 4 (1977) 135-140
59 N. Bernard, G. Cuisinaud and J. Sassard, J. Chromatogr., 228 (1982) 355-361
60 D.J. Miner, D.A. Binkley and L.D. Bechtol, Clin. Chem., 30 (1984) 717-723
61 K. Felder, H.E. Geißler, S. Hiemstra, E. Mutschler, M. Schäfer and E. Ziegler, Arzneim. Forsch., 29 (1979) 1746-1752
62 M. Schäfer-Korting and E. Mutschler, Eur. J. Clin. Pharmacol., 21 (1982) 315-323
63 S. Morita, M. Iinuma, M. Kido, S. Sakuragi, H. Kohri and H. Nishino, Arzneim. Forsch., 27 (1977) 2380-2383

64 T. Ishizaki, A. Ohnishi, T. Sasaki, K. Kushida, Y. Horai, K. Chiba and T. Suganuma, Eur. J. Clin. Pharmacol., 25 (1983) 95-101
65 S.Y. Chu, S.M. Vega, A. Ali and L.T. Sennello, J. Pharm. Sci., 70 (1981) 990-994
66 R. Chayen, S. Gould, A. Harell and C.V. Stead, Analyt. Biochem., 39 (1971) 533-535
67 G. Musumarra, G. Scarlata, G. Romano and S. Clementi, J. Anal. Toxicol., 7 (1983) 286-292
68 A.H. Stead, R. Gill, T. Wright, J.P. Gibbs and A.C. Moffat, Analyst, 107 (1982) 1106-1168

Chapter 6.1

IMMUNOLOGICAL METHODS FOR THE DETERMINATION OF BETA-BLOCKERS

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6.1.1 INTRODUCTION

   During the past 20 years, a variety of immunological methods
have been developed and used for the determination of drugs in the
tissues and biological fluids such as serum, plasma, saliva and
urine, because of their advantages of sensitivity, specificity,
precision and   simplicity of   operation (ref. 1). To date,
development of various types of immunoassays and their application
have been reported for 8 beta-blockers: propranolol (ref. 2-6),
acebutolol (ref. 7), oxprenolol (ref. 8), carteolol (ref. 9),
diacetolol (ref. 10), bunitrolol (ref. 11), befunolol (ref. 12)
and carazolol (ref. 13), in chronological order.   Beta-blockers
have an asymmetric carbon and have two stereoisomers: the $\underline{d}(+)$-
and $\underline{l}(-)$-isomers. Although the beta-blocking action can be mainly
attributed to the $\underline{l}$-isomer, in many cases, the racemates are
employed in clinical applications.   One of the   important
advantages of the immunoassay technique is that it enables us to
make use of the unique ability of the antibody to discriminate
between the stereo-isomeric forms, as demonstrated by Landsteiner
(ref. 14). This potential advantage has been realized in the
development of a stereospecific radioimmunoassay for propranolol
isomers (ref. 2). This stereospecific radioimmunoassay has been
applied to the determination of plasma or serum concentrations of
$\underline{dl}$- and $\underline{l}$-propranolol in rats (ref. 2), mice (ref. 15), rabbits
(ref. 16) and humans (ref. 17) after administration of the
racemate.
   The aims of this chapter are to briefly introduce the general

principles of immunoassays and to describe procedures for their
development and application. Furthermore, I will review the
immunoassays for specific beta-blockers, their applications to
the determination of the drugs in biological fluids, and discuss
their advantages and limitations.

6.1.2 PRINCIPLES

In an immunoassay, a substance (the antigen) is determined by
using its reaction with an antibody specific for the substance,
which leads to the formation of an antigen-antibody complex.
Thus, an antibody which reacts specifically with an analyte is one
of the critical components for an immunoassay, and the specificity
of the immunoassay largely depends on the cross-reactivities of
the antibody with the metabolites of the analyte and related
compounds.

Various types of immunoassays for many kinds of substances have
been developed specifically for a particular need and application.
However, these procedures can be simply classified into two
categories: the 'immunoassay' where a constant and a limited
amount of the antibody is used and the 'immunometric assay' where
a constant and an excess amount of the antibody is used. The
immunological assay methods for various beta-blockers developed so
far are of the immunoassay type (ref. 18). These immunoassays
depend on the competition between a limited amount of labeled
beta-blocker (Ag*) and the unlabeled drug (Ag) for combination
with the limited number of binding sites on the antibody molecule
(Ab).

```
        Ag                                              Ag-Ab
(unlabeled antigen)

              +

                          Ab

              +

        Ag*                                            Ag*-Ab
(labeled antigen)


        Free fraction                              Bound fraction
```
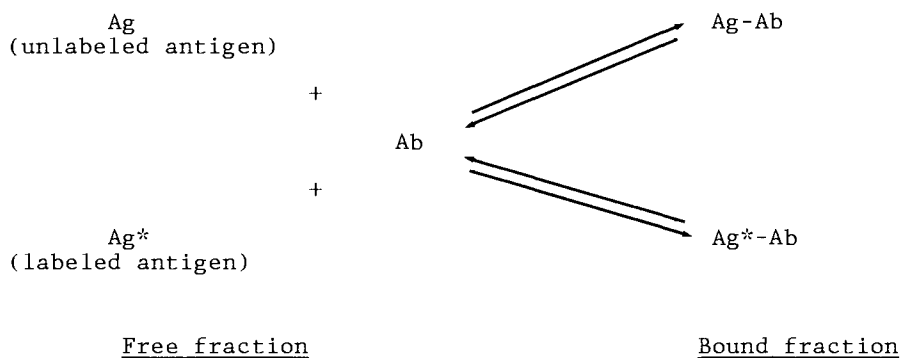
Distribution of the label at equilibrium either in the antibody-bound fraction or in the free fraction can be determined by separating the fractions using one of the appropriate methods (see TABLE 6.1.2). The percentage of the total label in the antibody-bound fraction will reflect and be inversely related to the total amount of antigen present in the reaction mixture. On the other hand, percent inhibition of the label-antibody binding will show a positive relationship with the total amount of antigen present in the assay tube. A standard curve is generated by adding known amounts of the standard drug to a reaction mixture of fixed composition containing the label and the antibody. Unknown quantities of the drug in the samples are determined by comparison with the standard curve.

6.1.3 DEVELOPMENT OF AN IMMUNOASSAY

Components required for the development of an immunoassay are listed in TABLE 6.1.1.

TABLE 6.1.1

Components required for the development of immunoassays

1. Immunogen
2. Antibody (Antiserum)
3. Label
4. Reaction Medium
5. Samples
6. Separation Technique, Bound/Free
7. Label Detection System

6.1.3.1 Preparation of Immunogens

Most beta-blockers which have molecular weight of less than 500 daltons are not immunogenic by themselves, like many other drugs. Therefore, it is necessary to prepare a proper immunogen which can stimulate an immune response and induce antibody production.

Usually, derivatives or analogs of beta-blocker, so called hap-
tens, are chemically coupled to carrier proteins with high molecu-
lar weights of more than 10,000 daltons and these conjugates are
used for the immunization. The antibodies produced in animals
immunized with the above immunogens will specifically react with
the respective beta-blockers.

(a) Carrier Proteins

Serum proteins such as albumin or gamma-globulin from various
species are used as carriers for the preparation of immunogens.
Sometimes, bovine or swine thyroglobulin and keyhole limpet
hemocyanin (KLH) may be used because of their strong immunogenic-
ity, but these proteins are difficult to handle because of their
poor solubility in aqueous solution. Bovine serum albumin (BSA)
is most commonly used for the synthesis of the immunogens of
beta-blockers, because of its low cost, ready availability, high
solubility in aqueous solution, and relative resistance to dena-
turation which may occur during the conjugation process. In most
cases, immunogens of beta-blockers are prepared by formation of an
amide linkage between carboxyl or amino groups of the haptens and
free amino or carboxyl groups in BSA and other carrier protein
molecules.

(b) Hapten Synthesis

Because beta-blockers lack the appropriate functional groups
for forming an amide linkage with BSA or other carrier proteins,
it is usually necessary to synthesize the haptens of beta-blockers
which have proper functional groups such as carboxyl and amino
groups. Furthermore, there should be careful planning about where
the functional group will be introduced into the molecule, because
the specificity of the antibody is generally directed primarily
against that portion of the molecule furthest from the site of
conjugation to the carrier protein (ref. 14). Thus, considerable
attention must be paid to the metabolic pathways of the drug and
to the preparation of the most suitable immunogen so as to obtain
an antibody which can distinguish between the parent drug and its
metabolites.

(c) Conjugation of Haptens to Carrier Proteins

Various methods are available for the conjugation of a hapten
to carrier proteins (ref. 19). An appropriate method should be

selected considering the chemical properties of the functional group and the stability of the hapten. The carbodiimide method and the mixed anhydride method are commonly used procedures for conjugation of haptenic drugs or drug derivatives to carrier proteins by formation of an amide linkage. Water-soluble carbodi-imides, such as 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDCI) and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide, have been the reagents of choice for coupling haptenic drugs with the carboxyl or amino groups to the carrier proteins. Although the coupling procedures with water-soluble carbodiimides are simple, the results of the coupling reaction with carbodiimides have been reported to be not always consistent. Conjugation of the haptenic drugs or drug derivatives with carboxyl function to carrier proteins will be carried out with much greater certainty by the mixed anhydride method.

6.1.3.2 Antibody (Antiserum)
(a) Immunization and Bleeding

To stimulate the immune response, immunogen dissolved or suspended in saline, buffer or distilled water is emulsified with complete Freund's adjuvant at a final concentration of 1 mg/ml or less. The emulsion is then injected intramuscularly, intradermal-ly, and subcutaneously into experimental animals, such as rabbits, guinea pigs, goats, or sheep. Although there is no standard immunization schedule, most commonly, immunization is repeated at every two weeks for the first several weeks and then once every month thereafter for up to 1 to 2 years. The blood samples are taken between one and two weeks after the booster immunization. Antiserum obtained from different animals and even that from the same animal at different bleeding times should not be pooled together, and must be kept separately, since the titer and speci-ficity of the antiserum will differ between the animals and during the immunization period. Antiserum of optimal titer and specific-ity will usually be obtained several months after the first immunization or later.

(b) Detection and Characterization of Antibodies

The presence of antibodies against the objective beta-blocker in the antiserum and their cross-reactivities with the metabolites must be determined before the development of the immunoassay. Since the sensitivity and specificity of an

immunoassay depend on the antiserum, the best antiserum should be
selected for the development of the assay.

(i) <u>Antibody Detection and Titration</u>. The presence of
antibodies in the antiserum can be easily determined by using the
labeled compound. A fixed volume of serially diluted antiserum is
reacted with a fixed amount of the labeled drug in the appropriate
medium. After incubation at room temperature or at 4°C for a
certain period of time, which should be sufficient for the binding
reaction between the label and the antibody to reach equilibrium,
the antibody-bound fraction of the label is separated from the
free fraction by an appropriate method. When the percentage of
the label found in the antibody-bound fraction with respect to the
total amount of the label added to the reaction mixture is plotted
against the magnitude of the antiserum dilution on a logarithmic
scale, one can find a sigmoidal antibody titration curve if
antibodies are present in the antiserum.

(ii) <u>Cross-Reactivities of the Antiserum</u>. A dilution of the
antiserum that will bind about 30 to 50% of the total amount of
the label added to the reaction medium should be selected for
further characterization of the antiserum. A fixed volume of the
diluted antiserum and a fixed amount of the label are incubated in
the presence and absence of various amounts of the drug or
metabolites. After separation of the label in the antibody-bound
from the free fractions, inhibition of the binding of the label to
the antibody by the standard drug or metabolites is calculated to
generate standard curves. The specificity of the antiserum is
evaluated by determining the amount of each compound required to
produce an inhibition of the label-antibody binding by 50%. The
cross-reactivity of each compound with the antiserum can be
expressed as percentage (X/Y x 100) of the 50% inhibitory amount
of the standard drug (X) to the 50% inhibitory amount of the
metabolites or related compounds (Y).

6.1.3.3 <u>Preparation of the Label</u>

Radioisotopes, fluorophore and enzymes have been used as label
in the development of immunoassays for beta-blockers.

(a) <u>Radioisotopes</u>

Tritium and iodine-125 (^{125}I) have been used to label beta-
blockers and their related compounds, with tritiation being the
method most commonly employed. Using the tritiated drugs,

radioimmunoassays have been developed for propranolol (ref. 2-4), acebutolol (ref. 7), diacetolol (ref. 10) and bunitrolol (ref. 11). ^{125}I-Labeled derivatives of carteolol (ref. 9) and carazolol (ref. 13) have been used for the development of the respective radioimmunoassays for the drugs.

(i) Tritium. Since the preparation of tritiated compounds requires special techniques and facilities, tritium labeled beta-blockers used for the development of radioimmunoassays are most often obtained from commercial sources or prepared at special laboratories. For example, tritiated dl-propranolol and l-propranolol with high specific activities can be purchased from NEN Research Products (U.S.A.) or Amersham International (England). Because the half-life of tritium is rather long (12.3 years), the tritium labeled compound can be used for a long period of time if it is chemically stable. However, since tritium must be detected by the liquid scintillation method, additional sample preparation is required. Thus, the use of tritium as a label for immunoassay incurs extra time and expense.

(ii) ^{125}I. Most beta-blockers can not be labeled directly with ^{125}I, and even if it is possible to do so, direct labeling of small compounds like beta-blockers with ^{125}I may affect its binding to the antibody. Thus, a derivative of carteolol that contains a phenolic group has been used for labeling with ^{125}I (ref. 9). The chloramine T method (ref. 20) has been most widely used for the preparation of ^{125}I-labeled derivatives of the drugs, and this has been used for the preparation of the ^{125}I-labeled derivative of carteolol. An ^{125}I-labeled compound usually has a higher specific activity than the corresponding tritiated compound and is suitable for the development of a sensitive radioimmunoassay. Although ^{125}I can be easily detected by placing samples in a gamma counter, it has only a short half-life (60 days) and labeled compound must be newly prepared every two to four months.

(b) Fluorescent Label

Fluorescein-labeled dl-propranolol has been prepared by reacting dl-propranolol with fluorescein isothiocyanate in the presence of triethylamine in methanol-water solution, and this derivative has been used to develop a fluoroimmunoassay (ref. 5).

(c) Enzyme Label

For the development of enzyme immunoassays for oxprenolol (ref. 8) and befunolol (ref. 12), beta-galactosidase has been used as an

enzyme label. Procedure similar to those used for the preparation
of conjugates of haptenic drugs to carrier protein have been
adopted for the preparation of enzyme labeled beta-blockers.
Although various enzymes have been used in the development of
enzyme immunoassays for other drugs (ref. 1, 18), mainly beta-
galactosidase is used for enzyme immunoassays of beta-blockers.
To develop a sensitive enzyme immunoassay, enzyme label with a
high specific activity is required. To attain this, a small
number of haptenic drug molecules should be coupled to one
molecule of enzyme. The mixed anhydride method is commonly used
for the preparation of enzyme label, because the number of
haptenic drug molecules coupled to each enzyme molecule can be
more easily controlled by this procedure than by the EDCI method.

6.1.3.4 Reaction Medium

   The medium most often used is phosphate-buffered saline, pH 6.6
to 7.4. Other media used for immunoassays of beta-blockers are
borate buffer, pH 9.0, and 0.1 M tris-HCl buffer, pH 7.4. When
the antiserum is diluted more than 100 times, the medium contain-
ing 0.1% lysozyme, 0.1% BSA, 0.1% gelatin, 0.1-0.3% gamma-
globulin, or 2.5% normal rabbit serum is used to prevent antibody
from adsorbing to the glass or plastic walls of the assay tubes.
Furthermore, if the separation is performed by precipitation of
the antibody using the ammonium sulfate method or the polyethylene
glycol method, a certain amount of gamma-globulin must be present
in the assay medium to induce coprecipitation of the antibody.
For such purposes, medium containing gamma-globulin or normal
rabbit serum would be suitable.

6.1.3.5 Samples

   Most often, 10 to 100 µl of unextracted serum or plasma
samples containing beta-blockers have been directly added to the
asssay tubes. The same volume of drug-free plasma or serum is
always added to the tubes for standards. In some instances, to
improve the specificity and sensitivity of the assay, extraction
of the drugs from the serum or plasma samples with an organic
solvent and even derivatization of the drugs may be required
before addition to the assay tubes (ref. 12).

6.1.3.6 Separation Technique

   After the incubation of the antiserum, the label, and sample or

standards in the assay tubes for a certain period of time, it is
necessary to separate the label in the antibody-bound fraction
from the free in most types of immunoassays. In the homogeneous
type of immunoassay, no separation procedure is necessary (ref.
6).

A wide variety of procedures are available for the separation
of the two fractions (ref. 19). Table 6.1.2 lists the separation
procedures used for immunoassays of beta-blockers.

TABLE 6.1.2

Techniques used for the separation of the antibody-bound and the
free fractions of labeled beta-blocker

Precipitation	1. Ammonium sulfate
	2. Polyethylene glycol (PEG)
Adsorption	1. Dextran coated charcoal (DCC)
Solid-phase antibody	1. Primary antibody coupled to magnetizable cellulose/iron oxide
	2. Goat anti-rabbit IgG
	a. Coupled to polyacrylamide gel
	b. Absorbed on polystyrene ball

(a) Ammonium Sulfate

The ammonium sulfate method (ref. 21) depends on the property
that antibodies belonging to the gamma-globulin will precipitate
in 50% saturated ammonium sulfate (SAS) solution. This has been
used frequently in radioimmunoassay since the method is simple,
and consistent results can be obtained by this procedure. Some-
times, however, a rather high value for the non-specific binding
is observed with this procedure, and this depends on the physico-
chemical nature of the label. Thus, washing of the precipitates
with 50% SAS solution is almost always required. Thus, additional
steps and extra time are needed for this procedure.

(b) Polyethylene Glycol

Polyethylene glycol (PEG) 6,000 is also used for the separation of the free and the antibody-bound fractions of the label in radioimmunoassay. This procedure depends on the property that antibody will precipitate in a solution of 5 to 20% PEG. Addition of gamma-globulin is required to induce coprecipitation of the antibody. Although the PEG method is rapid and simple, the results obtained by PEG method is likely to be a little bit less precise than those obtained by the ammonium sulfate method.

(c) Dextran Coated Charcoal

The dextran coated charcoal (DCC) method is also rapid and simple, and has been often used for radioimmunoassay. The proper amount of charcoal just sufficient to adsorb the label only in the free fraction is added to the incubation mixture. DCC is prepared by suspending the charcoal in the assay medium, and dextran is added in an amount of one-tenth that of charcoal to prevent the adsorption of the antibody to the charcoal. The precision of the results obtained by this method is almost comparable to that of the PEG method.

(d) Solid-Phase Antibodies

Primary or second antibodies coupled to solid-phase materials have been used in various kinds of immunoassays because separation can be performed easily (ref. 19). Primary antibodies against propranolol coupled to magnetizable cellulose/iron oxide particles have been used to develop a fluoroimmunoassay (ref. 5). Goat antibodies against rabbit IgG chemically coupled to polyacrylamide beads (Immunobeads®: Bio-Rad, U.S.A.) and adsorbed to polystyrene balls have been used for the development of enzyme immunoassays for oxprenolol (ref. 8) and befunolol (ref. 12), respectively. Solid-phase materials which have a specific gravity of slightly greater than 1, like Immunobeads® would be preferable, since they can float in the assay solution for a longer period of time without continuous agitation during the assay procedure.

6.1.3.7 Detection of the Label

After separation, the label either in the antibody-bound or the free fraction is detected by a variety of methods depending on the nature of the label (ref. 19).

(a) <u>Radioisotopes</u>

(i) <u>Tritium</u>. The tritiated label in the antibody-bound fraction has been determined by the liquid scintillation method. The precipitates formed by the 50% SAS and PEG methods are dissolved in a small volume of an appropriate solvent and then transferred into counting vials containing scintillation cocktail. After mixing, the radioactivities are measured in a liquid scintillation counter. When the DCC is used for the separation, the supernatant which contains the label in the antibody-bound fraction is dispensed into the counting vial and is then processed as described above.

(ii) <u>^{125}I</u>. In the case of an ^{125}I-labeled compound, radioactivity contained in either the supernatant or the precipitates can be directly determined in a gamma counter. Thus, the procedures for radioactivity determination are much simpler with ^{125}I than with tritium.

(b) <u>Fluorescence</u>

In fluoroimmunoassay for propranolol, fluorescein-labeled propranolol bound to the antibody has been determined in a fluorometer (ref. 5).

(c) <u>Enzyme</u>

In an enzyme immunoassay, enzyme activity located in the antibody-bound fraction is determined by reacting with the enzyme substrate. In enzyme immunoassays for oxprenolol (ref. 8) and befunolol (ref. 12), the enzyme activity of beta-galactosidase in the antibody-bound fraction is determined by incubating with the substrate 4-methylumbelliferyl-beta-D-galactoside, and the resultant fluorescence is then measured in a fluorometer.

In the homogeneous enzyme immunoassay (EMIT®) for propranolol (ref. 6), enzyme activity in the reaction mixture has been determined without requiring a separation procedure.

6.1.3.8 <u>Other Elements Required for Immunoassay of Beta-Blockers</u>

Small assay tubes (10 x 75 or 12 x 75 mm) made of glass or plastics, an appropriate incubation medium, specific antiserum at a proper dilution, milligram quantities of the pure drugs and metabolites, the label, a separation method, and a device for detection of the label are usually required to perform an immuno-

assay. Other elements required for an immunoassay for beta-
blockers which are not mentioned above will be briefly discussed
below.

(a) Amount of Label

    The proper amount of label to add to each assay tube depends on
the type of label. The assay will become less sensitive when too
much label is added to the assay tubes, while it will become less
precise when a too low amount is used for the assay. For example,
the amount of tritium labeled beta-blockers added to each assay
tube is usually between 7,500 and 15,000 dpm. In the cases of
^{125}I-labeled drugs, the amount of the label added per each assay
tube is between 10,000 to 20,000 dpm. Thus, selection of the
proper amount of label is based on the specific activity of the
label and the sensitivity and precision of the assay required for
the determination of the respective beta-blockers.

(b) Volume of Reaction Mixture

    The volume of a reaction mixture is usually between 0.5 to 1.0
ml. A too small volume makes the mixing of the contents in the
tube difficult, while a too large volume will affect the sensitiv-
ity of the assay.

(c) Incubation Period

    Since beta-blockers are small molecules, they can rapidly and
freely move in the assay medium and react with the antibody.
Thus, the binding reaction among the labeled drug, unlabeled drug
and the antibody will reach equilibrium within a short period of
time. However, for convenience, most often the contents of the
assay tube have been allowed to react at 4°C overnight. In some
cases, incubation of the reaction mixture has been carried out for
30 to 90 min at room temperature or 37°C and then 10 to 90 min at
0 or 4°C before separation.

6.1.4   IMMUNOASSAYS FOR SPECIFIC BETA-BLOCKERS

6.1.4.1 Propranolol

    Propranolol is a prototype non-cardioselective beta-blocker
with membrane stabilizing action. Although the d-isomer of

propranolol shares membrane stabilizing action, the l-isomer has about 100 times greater beta-blocking activity than the d-isomer. Three different types of immunoassays for dl-propranolol have been developed: radioimmunoassay (ref. 2-4), fluoroimmunoassay (ref. 5) and homogeneous enzyme immunoassay (ref. 6).

(a) Radioimmunoassays for dl- and l-Propranolol

Radioimmunoassays for dl-propranolol have been developed in three different laboratories using tritiated dl-propranolol as the label (ref. 2-4). In addition, development of a radioimmunoassay specific for l-propranolol has been reported by Kawashima et al. in 1976 (ref. 2). Specific features of these radioimmunoassays are summarized in TABLE 6.1.3.

(i) Procedures reported by Kawashima et al. (ref. 2).

Preparation of the immunogens. dl- or l-Propranolol was reacted with succinic anhydride in pyridine to form the hemi-succinate derivative of propranolol (Fig. 6.1.1). Although the resultant hemisuccinate derivative was thought to be O-hemi-succinyl propranolol at first, Nelson and Walker suggested later that the major product of the reaction between propranolol and succinic anhydride is the N-hemisuccinyl derivative (ref. 22). The hemisuccinate derivative of dl- or l-propranolol was conjugat-ed through the hemisuccinate carboxyl group to the amino groups of BSA by the mixed anhydride method (Fig. 6.1.1) (ref. 2).

Immunization. Conjugates of dl- or l-propranolol hemisuccinate to BSA were dissolved in phosphate-buffered saline and emulsified with an equal volume of complete Freund's adjuvant. The emulsion containing 500 μg of the immunogen was injected into the four foot pads and intramuscularly into both thighs of New Zealand white rabbits. Bleedings were taken from the central ear artery 6 to 8 days after the third booster injections.

Procedures for assay. A mixture of 400 μl of diluted antiserum in phosphate-buffered saline containing 2.5% normal rabbit serum, 10 μl of unknown or standard sample solution and 100 μl of triti-ated dl-propranolol solution (60 pg, about 7,500 dpm) in a glass assay tube (10 x 75 mm) was incubated at 4°C overnight. The antibody-bound label was separated from the free fraction of the label by the ammonium sulfate method (ref. 21). The radioactivity in the antibody-bound fraction was measured by the liquid scintil-lation method.

1) Hemisuccinate formation

Succinic anhydride

in pyridine

reflux

Propranolol (PPL)

PPL hemisuccinate
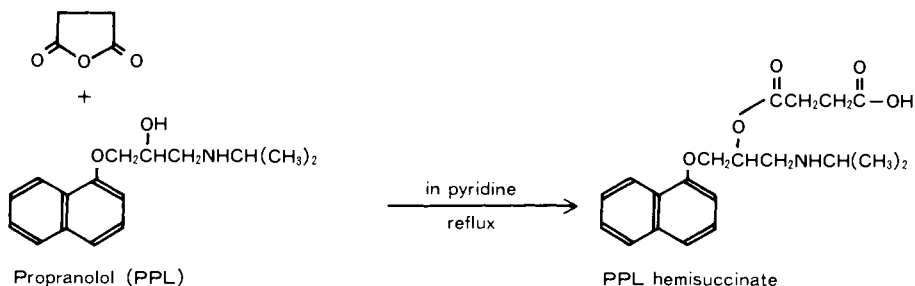
2) Conjugation to BSA (mixed anhydride method)

$PPL-O-\overset{O}{\overset{\|}{C}}CH_2CH_2\overset{O}{\overset{\|}{C}}-OH$ + $C_4H_9-O-\overset{O}{\overset{\|}{C}}-Cl$

isobutylchloroformate

$(C_2H_5)_3N$

in dioxane

$PPL-O-\overset{O}{\overset{\|}{C}}CH_2CH_2\overset{}{C}$

$O-\overset{}{C}-OC_4H_9$
$\overset{\|}{O}$

BSA$-NH_2$

in 50% dioxane

pH 9.0

$PPL-O-\overset{O}{\overset{\|}{C}}CH_2CH_2\overset{O}{\overset{\|}{C}}-NH-BSA$
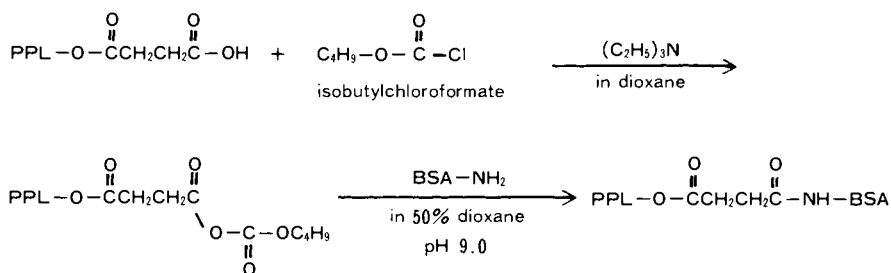
Fig. 6.1.1. Procedures for the preparation of propranolol immuno-
gen. PPL, propranolol; BSA, bovine serum albumin. Redrawn from
K. Kawashima et al. (ref. 2) by permission of Williams & Wilkins.

Specificity. Antiserum obtained from the rabbit immunized with
the conjugates of dl-propranolol hemisuccinate to BSA produced
antibodies that recognized both d- and l-propranolol to the same
degree, when tritiated dl-propranolol was used as the label. On
the other hand, the antiserum raised against l-propranolol immuno-
gen was able to selectively distinguish l-propranolol from the
d-isomer. The selectivity of the antiserum for the l-isomer was
further improved when tritiated l-propranolol, which is now
available from NEN Research Products, was used as the label
(unpublished data). These antisera crsoss-reacted with proprano-
lol glycol to a certain degree, but did not bind with other
side-chain metabolites to any significant extent. 4-Hydroxy-

propranolol, a ring hydroxylated metabolite, did not appreciably cross-react with these antisera (TABLE 6.1.3).

Sensitivities. Radioimmunoassays developed for dl- and l-propranolol using these antisera together with tritiated dl-propranolol (11 Ci/mmol) were able to detect as little as 10 pg/tube of the respective compounds (Fig. 6.1.2).
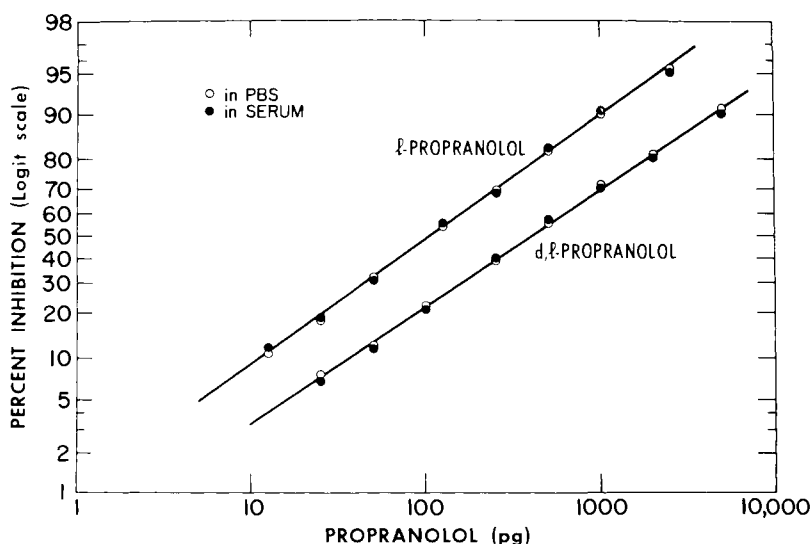
Fig. 6.1.2. Standard curves for dl- and l-propranolol. Percent inhibition of the label-antibody binding values by various amounts of unlabeled dl- and l-propranolol in phosphate-buffered saline and rat serum were transformed to logits and plotted against log amounts of unlabeled propranolol added. Reproduced from K. Kawashima et al. (ref. 2) by permission of Williams & Wilkins.

Specific applications of the stereospecific radioimmunoassays to the determination of dl- and l-propranolol in the plasma and serum of experimental animals and humans are discussed below.

(ii) Procedures reported by Mould et al. (ref. 3).

Preparation of immunogen. N-(4-bromobutyl)phthalimide was reacted with dl-propranolol to prepare N-(4-aminobutyl)propranolol (Fig. 6.1.3). Then, N-(4-aminobutyl)propranolol was conjugated through the amino group to the carboxyl groups of BSA by the EDCI method.
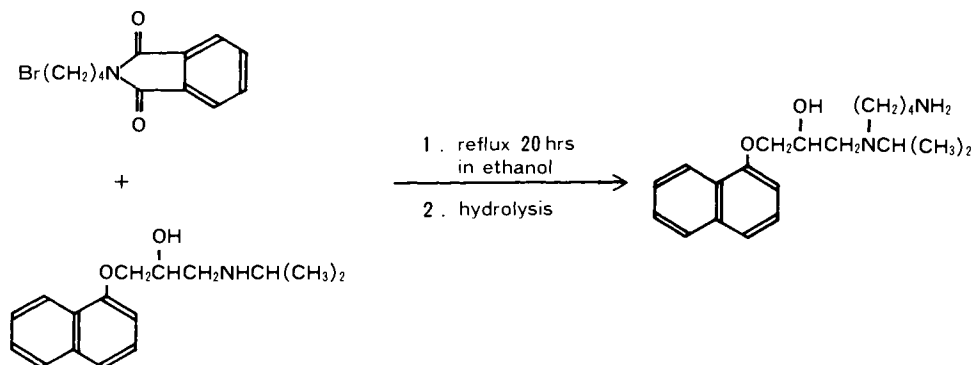
Fig. 6.1.3.   Procedures for the preparation of N-(4-aminobutyl)-propranolol (ref. 3).

Immunization.   Five sheep were immunized with the conjugates of N-(4-aminobutyl)propranolol to BSA dissolved in saline and emulsified with complete Freund's adjuvant.   Booster doses of the immunogen were given about one year after the first immunization at one to 3 month intervals.   Samples of blood were taken at regular intervals to obtain the antisera.

Procedures for assay.   Phosphate buffer (0.05 M, pH 7.4) containing 0.1% gelatin and   0.6% NaCl was used for the assay. Tritiated dl-propranolol (23 Ci/mmol) was used as the tracer for this radioimmunoassay. A mixture of 50 μl of sample plasma or standard plasma, 100 μl of the diluted antiserum, 100 μl of the label and assay buffer to bring the total volume up to 500 μl in a plastic tube was incubated at room temperature for 30 min and at 4°C for 10 min.   The label in the antibody-bound fraction was separated from the free label by the addition of DCC and centrifugation.   A 200-μl portion of the supernatant was mixed with 4.8 ml of scintillation fluid and measured for radioactivity in a liquid scintillation counter.

Specificity. Cross-reactivities of a suitable antiserum with the optical isomers of propranolol and 4-hydroxy-propranolol are shown in TABLE 6.1.3.   The antiserum   reacted with dl-, d- and l-propranolol to the same degree.   In other words, the antiserum did not show any stereospecificity.   Cross-reactivity of the antiserum with 4-hydroxy-propranolol was comparable to those

reported by Kawashima et al. (ref. 2) (TABLE 6.1.3). No data on the cross-reaction with side-chain metabolites were reported.

Sensitivity. A radioimmunoassay developed with the antiserum and the tritiated dl-propranolol was able to detect as little as 615 pg/ml of propranolol in unextracted plasma using a 50 μl sample.

Precision and reproducibility. Average recovery of propranolol added to drug-free plasma pools was 95 ± 4.9% (mean ± S.E.M.). Inter-assay precision was between 2.7 and 15.4% at various plasma propranolol concentrations. Radioimmunoassay results on plasma samples to which propranolol had been added correlated well with those obtained with fluorometric methods (r = 0.97) and gas liquid chromatography (r = 0.99).

(iii) Procedures reported by Eller et al. (ref. 4)

Preparation of immunogens. Eller et al. (ref. 4) have prepared 5'-, 7'-, and 8'-(3-aminopropoxy)propranolol derivatives that have a functional group in the naphthalene ring moiety of the compound (Fig. 6.1.4). These derivatives were conjugated to succinylated BSA by the EDCI method.

Immunization. Respective immunogens were dissolved in phosphate buffer and emulsified with complete Freund's adjuvant, and injected into New Zealand white rabbits. After repeated booster immunization, antisera were obtained from these rabbits.

Specificity. Phosphate-buffered saline, pH 7.4, was used for the assay. Cross-reactivities of the antisera with various metabolites of propranolol were determined using tritiated dl-propranolol (24 Ci/mmol) as the label. The antibody-bound fraction of the label was separated from the free label by the DCC method. The antisera cross-reacted with 4-hydroxy-propranolol, a ring oxidized metabolite, to a certain degree, but did not react with side-chain metabolites (TABLE 6.1.3). These results are different from those reported on the two previous antisera (ref. 2 and 3). No data were available on the specificity of these antisera towards the d- and l-isomers of propranolol.

Sensitivity. The radioimmunoassay developed with the antiserum against the conjugates of 5'-(3-aminopropoxy)propranolol derivative to succinylated BSA and tritiated dl-propranolol had a sensitivity of 20 pg/tube of dl-propranolol in a toluene extract of tissue or plasma. For the majority of the plasma samples, only 50 μl of the extract was required for the determination.
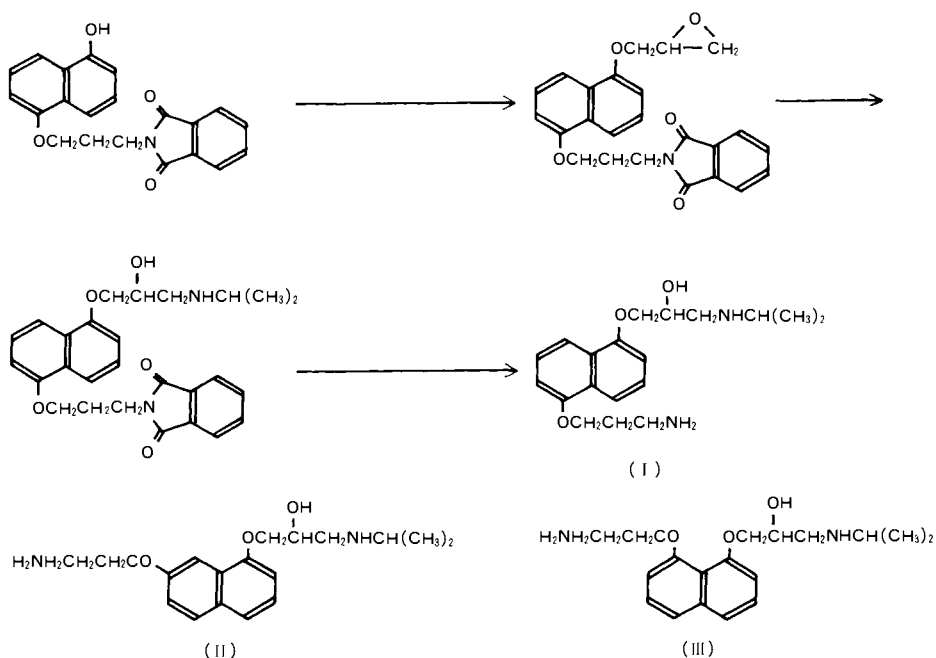
Fig. 6.1.4. Procedures for the preparation of 1-(2-propylamino)-3-[5-(3-aminopropoxy)naphthalenyloxy]-2-propanol (I), and structures of 7'- and 8'-(3-aminopropoxy)propranolol derivatives (II and III). Redrawn from T.D. Eller et al. (ref. 4) with permission from the Analytical Chemistry.

Precision and reproducibility. Inter-assay and intra-assay precisions of this procedure were reported to be 5 and 4%, respectively, at the midpoint of the standard curve (about 500 pg/tube). The results obtained by the radioimmunoassay in toluene extracted dog and human plasma samples correlated well with those obtained by gas chromatography/mass spectrometry (GC/MS) (r = 0.99). However, the direct radioimmunoassay of human plasma using up to 50 μl of sample gave about 10-fold higher values than those obtained by GC/MS, indicating that unextracted plasma contains high levels of metabolites which cross-react with the antiserum, and these are eliminated by the extraction procedure.

The titers of the antisera against the conjugates of 7'- and 8'-(3-aminopropoxy)propranolol derivatives to succinylated BSA were rather weak in spite of excellent specificity. Furthermore,

TABLE 6.1.3

Specific Features of Radioimmunoassays for dl- and l-Propranolol

Reference	Kawashima et al. (ref. 2)		Mould et al. (ref. 3)	Eller et al. (ref. 4)		
Haptenic drug	dl-HS-PPL	l-HS-PPL	dl-NAB-PPL	dl-5AP-PPL	dl-7AP-PPL	dl-8AP-PPL
Sensitivity	10 pg/tube	10 pg/tube	615 pg/ml	20 pg/tube	NR	NR
Cross-reactivity (%)						
dl-propranolol	NR	NR	100	100	100	100
d-propranolol	100	7.2	100	NR	NR	NR
l-propranolol	102.2	100	100	NR	NR	NR
4-hydroxy-propranolol	1.5	2.9	2	59	1.9	4.7
naphthoxylactic acid	NR	NR	<0.1	NE	0.01	0.001
1-naphthoxyacetic acid	<0.3	<0.1	NR	NR	NR	NR
1,4-naphthalene-diol	<0.3	<0.1	NR	NR	NR	NR
1-naphthol	<0.3	<0.1	NR	NR	NR	NR
propranolol glycol	7.3	10.8	NR	NE	0.15	0.007
propranolol glucuronide	NR	NR	NR	0.08	0.07	0.2
N-desisopropyl-propranolol	NR	NR	NR	0.06	2.5	0.4

HS-PPL, hemisuccinyl-propranolol; NAB-PPL, N-aminobutyl-propranolol; AP-PPL, aminopropoxy-propranolol;
NR, not reported; NE, no effect. Tritiated dl-propranolol was used as the label.
Adapted from K. Kawashima et al. (ref. 2), G.P. Mould et al. (ref. 3) and Eller et al. (ref. 4) with permission
of Willams & Wilkins, John Wiley & Sons, Ltd. and The American Chemical Society.

radioimmunoassays with these antisera were less sensitive than
that described above.

(b) Fluoroimmunoassay for dl-Propranolol

The development of a fluoroimmunoassay for the determination of
serum or plasma dl-propranolol has been reported using anti-pro-
pranolol antibodies coupled to magnetizable solid-phase particles
and fluorescein-labeled propranolol as the tracer (ref. 5).

Preparation of immunogen. The conjugates of dl-propranolol
hemisuccinate derivative to BSA were prepared by the method shown
above (Fig. 6.1.1) (ref. 2).

Immunization. The immunogen was injected into sheep to obtain
the antiserum.

Preparation of solid-phase reagent. Whole antiserum was coupled
to magnetizable cellulose/iron oxide particles using the cyanogen
bromide method (ref. 23).

Procedures for assay. Assay reagents were dissolved or sus-
pended in sodium borate buffer (400 mM, pH 9.0) containing 2g/l
sodium salicylate to prevent the non-specific binding of
fluorescein-labeled propranolol by serum or plasma, 1 ml/l Triton
X-100, and 1 g/l sodium azide. In disposable polystyrene test
tubes, a mixture of 100 μl of serum or plasma sample, 100 μl of
fluorescein-labeled propranolol and 100 μl of anti-propranolol
solid-phase reagent was incubated at room temperature for 1 hr on
a mechanical shaker. Then, 1 ml of diluent buffer was added. The
particles were sedimented by placing the test tubes on a magnet,
and the supernatant was aspirated to waste. Elution reagent
consisting of a mixture of methanol and sodium bicarbonate buffer
(50 mM, pH 9.0) in the proportion of 7:3 by volume was added to
the tubes. Then, the tubes were placed in a fluorometer to
measure the fluorescence of the label in the antibody-bound
fraction.

Sensitivity. A standard curve was obtained that covered the
clinically significant range of plasma propranolol concentrations
(10 to 250 ng/ml).

Precision. Intra-assay and inter-assay precisions determined
in patient's sera (ranged 35 to 140 ng/ml) were 3.8-5.6% and
5.2-8.8%, respectively.

Specificity. The antiserum used for the development of the
fluoroimmunoassay cross-reacted with d-, l-, and dl-propranolol to
the same degree, and it did not show any stereospecificity.

Cross-reactivities of the antiserum with 4-hydroxy-propranolol and N-desisopropyl-propranolol were 4 and 16%, respectively. Thus, the specificity of the antiserum was quite similar to that used for the development of the radioimmunoassay described above (ref. 2 and 3) (TABLE 6.1.3).

Reproducibility. Results of the fluoroimmunoassay on serum or plasma samples from patients given dl-propranolol correlated well with those obtained by the established fluorometric method (ref. 24) (r = 0.99). The good correlation of the fluoroimmunoassay with the fluorometric assay indicates the specificity of the method, and it suggests that when the antiserum raised against the side-chain derivatives of propranolol is used for the assay, both side-chain and ring oxidized metabolites do not interfere significantly with the assay even when they may present in much higher concentrations than propranolol itself.

(c) Homogeneous Enzyme Immunoassay for dl-Propranolol

A homogeneous type of enzyme immunoassay (EMIT$^{\circledR}$) for dl-propranolol in serum has been reported by Chegwidden et al. (ref. 6). In this type of immunoassay, enzyme activity is reduced whenever the enzyme-labeled propranolol is bound to the antibody. Thus, the separation of the label bound to the antibody from the free label is not required.

Sensitivity. The sensitivity of the assay was 25 ng/ml.

Specificity. Metabolites of propranolol such as 4-hydroxy-propranolol, N-desisopropyl-propranolol, propranolol glycol, naphthoxylactic acid, naphthoxyacetic acid, and 1-naphthol have been found not to interfere with the assay.

Precision. The coefficient of variation of replicates of the 100 ng/ml calibrator was less than 15%.

One of the advantages of this procedure over fluorometry, GLC and HPLC is that it requires no extraction of the serum sample.

6.1.4.2 Radioimmunoassays for Acebutolol and Diacetolol

Acebutolol, dl-1-(2-acetyl-4-butyramidophenoxy)-2-(3-carboxy-propionyloxy)-3-isopropyl-aminopropane, is a cardioselective beta-blocker with intrinsic sympathomimetic (partial agonist) and membrane stabilizing activities, and it is metabolized into three compounds. One of the metabolites, diacetolol, dl-1-(2-acetyl-4-acetamidophenoxy)-2-hydroxy-3-isopropyl-aminopropane has anti-arrhythmic and beta-blocking properties resembling those of

acebutolol (ref. 25). Development of radioimmunoassays for acebutolol and diacetolol has been reported by Gourmel et al. (ref. 7 and 10).

(a) Acebutolol (ref. 7)

Preparation of the immunogen. Acebutolol was reacted with succinic anhydride to form acebutolol hemisuccinate in dry pyridine. Acebutolol hemisuccinate was conjugated to BSA by the EDCI method. The number of acebutolol molecules per BSA molecule was revealed to be 2 after hydrolysis and spectrophotometric assay.

Immunization. New Zealand white rabbits were immunized with acebutolol hemisuccinate-BSA (0.5 mg), which was suspended in complete Freund's adjuvant and then administered intradermally at 40-50 sites on the shaved back. Booster injections were repeated monthly for one year. Antisera were obtained on the 10th day after the booster injections.

Procedures for radioimmunoassay. Tritiated dl-acebutolol (59.6 Ci/mmol) and the antisera were used for the development of the radioimmunoassay. Phosphate buffer (0.04 M, pH 7.4) containing 0.1% gelatin and 0.1% sodium azide was used for the assay. To glass assay tubes containing diluted antiserum, tritiated acebutolol (about 5000 dpm) and diluted samples, assay buffer was added to bring the total volume up to 0.7 ml, and then the mixture was incubated for 30 min at 37°C and left for 1.5 hr at 4°C for equilibration. The antibody-bound tritiated acebutolol was separated from the free fraction by the addition of 0.5 ml of the DCC (0.25%).

Specificity. The antiserum did not cross-react with metabolites and other beta-blockers to any significant degree (up to 1%). Furthermore, the value determined by this radioimmunoassay for a sample containing acebutolol and diacetolol, the major metabolite of acebutolol, in normal untreated plasma was not significantly different from that for acebutolol alone. The results indicate that this radioimmunoassay is specific for acebutolol. Cross-reactivities of the antiserum with d- and l-acebutolol were not determined.

Sensitivity. The lowest detectable dose of acebutolol was 10 pg/tube. If 100 µl of diluted plasma (1:10) sample is used for the assay, the lowest detectable dose of acebutolol in plasma will be 1 ng/ml.

Reproducibility. Intra-assay variations were 4.23% at 600 ng/ml. Inter-assay variations of 6.33 and 6.96% were observed on two different occasions.

Plasma concentrations of acebutolol in hypertensive patients receiving two 400 mg doses of acebutolol at 08.00 and 19.00 hr were determined by this radioimmunoassay. The mean peak plasma concentration in these patients was 1470 ng/ml.

(b) Diacetolol (ref. 10)

Preparation of the immunogen. The diacetolol hemisuccinate-BSA conjugate was prepared by a method similar to that used for the preparation of acebutolol immunogen (ref. 7). Antisera against diacetolol were obtained following the same procedure as that used to raise antisera against acebutolol.

Procedures for assay. Tritiated diacetolol with a specific activity of 4.25 Ci/mmol was used as the tracer. A mixture of diluted sample or standard solution and the diluted antiserum in a disposable glass tube was incubated for 30 min at 37°C. Then, 0.1 ml of the tracer (5000 dpm) was added to the tube and the mixture was incubated further for 30 min at 4°C for equilibration. The antibody-bound tracer was separated from the free label by the DCC method. The radioactivity in the antibody-bound fraction was measured in a liquid scintillation counter.

Specificity. Cross-reactivities of the antiserum with acebu-tolol, metabolite II and metabolite III were less than 0.6%. No cross-reactions were observed with other beta-blockers. Cross-reactivities with d- and l-diacetolol were not tested.

Sensitivity. The lowest detectable dose was 35 pg/tube. If the assay is performed with 0.2 ml of diluted plasma sample (1:10 dilution), the limit of the sensitivity can be 1.75 ng/ml.

Reproducibility. Intra-assay covariations at 400 ng/ml and 1200 ng/ml were 5.12% and 4.90%, respectively. Inter-assay covariations at 400 ng/ml and 1200 ng/ml were 7.3% and 4.3%, respectively. The results of radioimmunoassays on plasma diaceto-lol in 45 samples correlated well with those obtained by HPLC coupled with spectrofluorometry (ref. 26) (r = 0.989).

Plasma concentrations. Plasma concentrations of diacetolol were determined in samples obtained from 4 subjects administered a single oral dose of 400 mg acebutolol. The maximum diacetolol concentration was observed 4-5 hr after the administration, and it varied between 513 ng/ml and 1390 ng/ml.

The radioimmunoassay for plasma diacetolol appears to have excellent specificity and reproducibility. Because it requires no prior extraction or concentration steps, this procedure would be suitable for routine clinical assay of diacetolol in plasma. As a result of the high sensitivity of the method, only a small volume of plasma sample is required for the determination of diacetolol.

6.1.4.3 Enzyme Immunoassay for Oxprenolol

Oxprenolol is a non-cardioselective beta-blocker with an intrinsic sympathomimetic activity. A heterogeneous type of enzyme immunoassay has been reported for oxprenolol (ref. 8).

Preparation of immunogen. dl-Oxprenolol was reacted with glutaric anhydride in triethylamine to form dl-oxprenolol hemiglutarate. dl-Oxprenolol hemiglutarate was conjugated to BSA using EDCI.

Immunization. The immunogen was dissolved in distilled water and emulsified with an equal volume of complete Freund's adjuvant. The emulsion containing 1 mg of the immunogen was injected into the four foot pads and subcutaneously into several sites on the back of rabbits. Bleedings were taken from the central ear artery 7 to 10 days after the booster injections.

Preparation of the label. dl-Oxprenolol hemisuccinate was prepared from dl-oxprenolol and succinic anhydride by the same procedures as used for the synthesis of the hemiglutarate derivative. dl-Oxprenolol hemisuccinate was conjugated to beta-galactosidase by the mixed anhydride method.

Buffer. Phosphate buffer (0.01 M, pH 7.0) containing 0.1% BSA, 0.001 M $MgCl_2$, and 0.1 M NaCl was used as the working buffer for this enzyme immunoassay.

Procedures for enzyme immunoassay. A mixture of 100 μl of diluted antiserum, 50 μl of the label (0.5 μU), 20 or 40 μl of sample plasma and a sufficient volume of working buffer to bring the final reaction volume up to 350 μl in a 12 x 70 mm plastic tube was incubated overnight at 4°C. To separate the antibody-bound and the free fractions of the label, Immunobeads[®] (Bio-Rad, U.S.A.) with goat anti-rabbit immunoglobulins were added to the tube and incubated for 2 hr at 30°C with occasional mixing. After centrifugation, the supernatant was discarded by aspiration. The precipitate containing the antibody-bound fraction of the

label was suspended in 800 µl of working buffer. The tubes were
added with 5 µg of 4-methylumbelliferyl-beta-D-galactoside, the
substrate for beta-galactosidase, in 400 µl and incubated for one
hr at 30°C with occasional mixing. The reaction was terminated by
the addition of 3 ml of ice-cold 0.1 M glycine-NaOH buffer, pH
10.5. Fluorescence intensity in the supernatant was measured in a
fluorometer.

Sensitivity. An enzyme immunoassay developed with the specific
antiserum and the beta-glactosidase-labeled dl-oxprenolol has the
sensitivity of one ng/tube, which is comparable to those of GLC
(ref. 27) and TLC (ref. 28). The standard curve was linear up to
300 ng/tube when plotted on logit-log scale.

Specificity. Cross-reactivity of the antiserum with N-dealkyl-
ated oxprenolol was about 1.7%. Considering the degree of cross-
reactivity of N-dealkylated oxprenolol and the chemical structure
of the immunogen, ring hydroxylated and O-dealkylated metabolites
do not appear to cross-react extensively with the antiserum. The
antiserum did not cross-react with other beta-blockers to any
significant degree. Cross-reactivities of the antiserum with d-
and l-oxprenolol were not tested.

This enzyme immunoassay has distinct advantages over other
procedures in that dl-oxprenolol can be measured directly from a
small volume of plasma without the need for extraction and deriva-
tive formation. With this procedure, plasma concentrations of
dl-oxprenolol in spontaneously hypertensive (SHR) rats were
determined after single and multiple oral doses, and no signifi-
cant differences in pharmacokinetic parameters were observed
between the treatments (ref. 8).

6.1.4.4 Radioimmunoassay for Carteolol

Carteolol is a non-cardioselective beta-blocking drug with an
intrinsic sympathomimetic activity. Development of a radioimmuno-
assay for carteolol in human plasma has been reported by Chu et
al. (ref. 9).

Preparation of immunogen. Carteolol was reacted with glutaric
anhydride in pyridine to form O-glutarylcarteolol. Then, O-
glutarylcarteolol was conjugated to BSA by the EDCI method (Fig.
6.1.5). The number of haptenic molecules conjugated to one BSA
molecule was estimated to be around 19 by UV absorption.

250
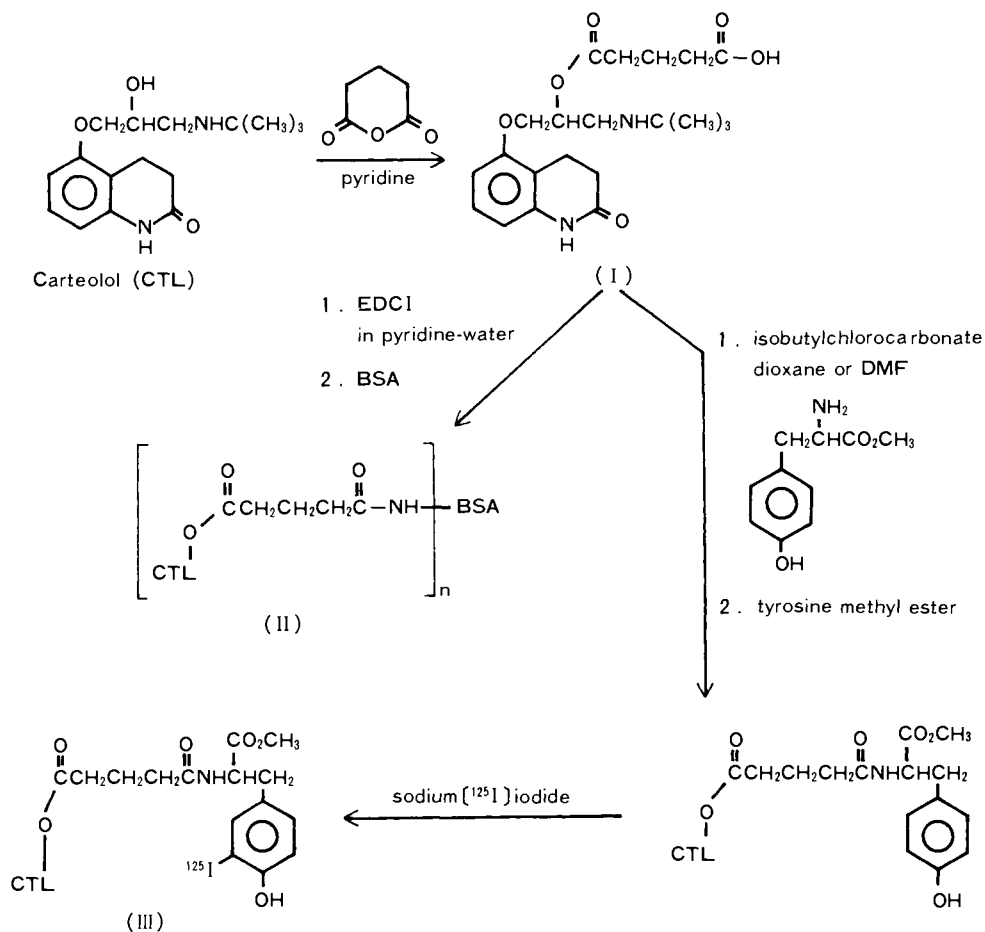
Fig. 6.1.5. Procedures for the preparation of carteolol immunogen (II) and Iodine-125-labeled radioactive tracer (III). CTL, carteolol. Redrawn from S.-Y. Chu et al. (ref. 9) with permission of the copyright owner, the American Pharmaceutical Association.

Immunization. The immunogen was dissolved in physiological saline at a concentration of 1 mg/ml and emulsified with an equal volume of complete Freund's adjuvant. New Zealand white rabbits were immunized by injecting 1 ml of the emulsion containing 0.5 mg of the immunogen into four footpads and two thighs. Booster injections were repeated once a week for 4 weeks and then once every 2-6 weeks. To obtain the antisera, rabbits were bled by cardiac puncture 6 to 14 days after the booster injection.

Preparation of [125]I-labeled antigen. The O-glutarylcarteolol-tyrosine methyl ester conjugate was synthesized by reacting O-glutarylcarteolol with tyrosine methyl ester by the mixed anhydride method. Then, O-glutarylcarteolol tyrosine methyl ester was reacted with sodium [[125]I]iodide by the chloramine-T method to form [[125]I]-O-glutarylcarteolol tyrosine methyl ester (Fig. 6.1.5) (ref. 20).

Procedures for immunoassay. Phosphate-buffered saline (0.01 M, pH 7.4) containing 0.1% gelatin and thimerosal was used as the working solution. A mixture of 100 μl of the diluted antiserum solution, 100 μl of sample or standard plasma, 200 μl of gelatin buffer and 100 μl of the [125]I-labeled antigen solution was incubated at 4°C overnight. The antibody-bound label was separated from the free fraction by the addition of 1.5 ml of an 18% solution of PEG 6000. The radioactivity of the antibody-bound fraction was counted in a gamma counter.

Sensitivity. The radioactivity counts were divided by the counts from control plasma. A calibration curve was constructed using the mean ratios of percent bound versus the logarithms of plasma carteolol concentrations. The lowest detectable concentration of carteolol was around 0.4 ng/ml using 0.1 ml of plasma sample.

Specificity. Although dehydrocarteolol cross-reacted with the antiserum to a certain degree (about 1.5%), this compound is not a metabolite of carteolol. Cross-reactivity of the antiserum with the major metabolite, 8-hydroxy-carteolol was less than 0.2%. Furthermore, available data indicate that plasma 8-hydroxy-carteolol concentrations within 12 hr after oral administration are quite low compared with the corresponding parent drug concentrations. Thus, the metabolite should not affect the determination of carteolol concentrations of clinical samples by this procedure. Cross-reactivity of the antiserum with d- and l-carteolol has not been reported.

Reproducibility. The daily replicate curves were quite reproducible, and the relative standard deviations (n = 3) were mostly within ±8%. The averages of the assayed carteolol concentrations from the nine standard curves were in good agreement with the theoretical concentrations, all being within 90-113% of the theoretical values.

The radioimmunoassay for carteolol developed using [125]I-labeled tracer is quite specific and has good intra-assay and inter-assay

reproducibilities. Since it does not require solvent extraction
or concentration processes, it has the advantage of simplicity of
operation. In addition, the radioimmunoassay for carteolol offers
sensitivity that is far superior to the other available methods.

6.1.4.5 Radioimmunoassay for Bunitrolol

Bunitrolol is a non-cardioselective beta-blocker with a
sympathomimetic action. Development of a radioimmunoassay for
dl-bunitrolol has been reported using the tritiated label (ref.
11).

Antisera. The immunogens for dl- and l-bunitrolol were synthe-
sized by the same procedures as used for the preparation of the
oxprenolol immunogen (ref. 8). To obtain the antisera, rabbits
were immunized with either the conjugate of dl- or l-bunitrolol
hemiglutarate to BSA.

Specificity. The antiserum against dl-bunitrolol bound with
both d- and l-bunitrolol almost to the same degree. The antiserum
against l-bunitrolol did not show any stereospecificity. The
metabolites of bunitrolol, 4-hydroxy-bunitrolol and O-nitrilo-
phenoxylactic acid did not show any appreciable cross-reactivities
with both the antisera.

Procedures for assay and sensitivity. The antiserum obtained
from a rabbit immunized with dl-bunitrolol immunogen and tritiated
dl-bunitrolol with a specific activity of 28 Ci/mmol were used for
the development of the radioimmunoassay. Tris-HCl buffer (0.1 M,
pH 7.4) was used as the working solution. A mixture of 100 μl of
diluted antiserum, 100 μl of tritiated bunitrolol solution con-
taining 42.1 pg (about 9,000 dpm), 20-50 μl of the diluted plasma
sample (1:10 dilution) and sufficient working solution to bring
the total volume up to 500 μl in a 10 x 75 mm glass tube was
incubated overnight at 4°C. The antibody-bound label was sepa-
rated from the free label by the ammonium sulfate method (ref.
21). The radioactivity in the antibody-bound fraction was
measured by the liquid scintillation method. The sensitivity of
this procedure was 100 pg/tube.

Concentrations of dl-bunitrolol were determined by radioimmuno-
assay directly from plasma without the need for extraction in SHR
rats after a single oral administration of 5 mg/kg dl-bunitrolol.
Bunitrolol was rapidly absorbed from the gastrointestinal tract
and the elimination half-life was found to be 1.8 hr in the SHR
(ref. 29).

6.1.4.6 <u>Enzyme Immunoassay for Befunolol and its Metabolite (M1)</u>

Befunolol, <u>dl</u>-2-acetyl-7-(2-hydroxy-3-isopropylaminopropoxy)-benzofuran, is a non-cardioselective beta-blocker with an intra-ocular pressure decreasing action. Enzyme immunoassays for befunolol and its metabolite (M1), <u>dl</u>-2-(1-hydroxyethyl)-7-(2-hydroxy-3-isopropylaminopropoxy)benzofuran, have been reported by Sato and Yamamoto (ref. 12).

<u>Preparation of the immunogens</u>. Befunolol was reacted with succinic anhydride in the presence of triethylamine to form N-succinylbefunolol. N-Succinylbefunolol was conjugated to human serum albumin (HSA) by the mixed anhydride method. The number of befunolol molecules conjugated to one HSA molecule was estimated to be 6.

N-Succinyl M1 was prepared by reduction of N-succinylbefunolol with sodium borohydride and then coupled to HSA with the mixed anhydride method. The number of M1 molecules coupled to one HSA molecule was estimated to be 2.8.

<u>Immunization</u>. The immunogens were mixed in complete Freund's adjuvant and injected intradermally into male albino rabbits. Booster injections were repeated every four weeks. Bleedings were taken from the ear vein 7 to 10 days after the booster injections.

<u>Preparation of the labels</u>. N-Succinylbefunolol or N-succinyl M1 (5 mg) was conjugated to beta-galactosidase (500 μg) by the mixed anhydride method. The numbers of befunolol and M1 molecules coupled to one beta-galactosidase molecule were estimated to be 7 and less than 3, respectively.

<u>Preparation of solid-phase second antibody reagent</u>. Solid-phase second antibody reagent was prepared by soaking polystyrene balls (1/4 in. diameter) in a solution of goat IgG fraction containing anti-rabbit IgG antibody in phosphate buffer (0.05 M, pH 7.5) for 24 hr at 4°C.

<u>Procedures for sample preparation</u>. Extraction of befunolol and M1 from plasma by acetone before enzyme immunoassay is required because of the presence of interfering substances in plasma. A 200-μl portion of plasma was mixed with 400 μl of cold acetone and placed in an ice-bath. After centrifugation, 300 μl of the supernatant was transferred to another tube, and this was reacted with 100 μl of succinylating reagent (1:9 v/v mixture of 20 g/l of succinic anhydride in triethylamine and acetone) for one hr at room temperature. The contents of the tubes were dried under vacuum and dissolved in 500 μl of the working solution.

Procedures for assay. A mixture of 200 μl of succinylated standard or sample, 100 μl of the label and 100 μl of the diluted antiserum was incubated for 18 hr at 4°C. The antibody-bound label was separated from the free fraction by adding the solid-phase second antibody and shaking for 5 hr at room temperature. The enzyme activity in the antibody-bound fraction was determined by procedures similar to those used for oxprenolol assay as described above (ref. 8).

Sensitivity. The lowest detectable doses of befunolol with and without the succinylation process were 0.3 and 30 pg/tube, respectively. In the Ml assay, the lowest detectable doses with and without the succinylation process were 3 and 100 pg/tube, respectively.

Specificity. Cross-reactivities of the antiserum against befunolol with Ml and succinylated Ml were 1.6 and 0.04%, respectively. Other metabolites such as 2-O-glucuronyl Ml, 4-hydroxy-befunolol (M2) and 4-O-glucuronyl M2 did not cross-react with the antiserum to any significant degree (< 0.1%). Cross-reactivities of the antiserum with the d- and l-isomers of befunolol were not tested.

Cross-reactivities of the antiserum against Ml with befunolol and succinylated befunolol were 1.3 and 0.13%, respectively. Other metabolites did not cross-react with the antiserum to any significant extent (< 0.1%)

Precision and reproducibility. Recovery ratios of befunolol and Ml added to standard plasma throughout the procedures were 100.6 and 100.8%, respectively. Intra-assay variations for befunolol and Ml assays were 2.0-5.1 and 3.0-9.7%, respectively. Inter-assay variations for befunolol and Ml assays were 5.3-6.8 and 6.8-14.9%, respectively. Concentrations of befunolol in rabbit plasma determined by this procedure correlated well with those measured by HPLC (r = 0.994). However, plasma concentrations of Ml determined by the enzyme immunoassay correlated poorly with those measured by HPLC (r = 0.727).

Application. Since befunolol has an intraocular pressure decreasing activity, one of the clinical applications of this drug is for the treatment of glaucoma. Thus, plasma concentrations of befunolol were determined by this enzyme immunoassay after one drop instillation (50 μl of 0.2, 1.0 and 5.0% solution) into the eye of the rabbit. Befunolol was detected in the plasma up to 6 hr by this procedure even after the instillation of the lowest

dose. M1 was also detected by the enzyme immunoassay in the above samples up to 6 hr after the instillation. The peak plasma concentration of M1 was observed 30 min after the instillation.

Although succinylation of befunolol and its metabolite (M1) before the assay improve both the sensitivity and specificity, additional steps of extraction and succinylation are required in the proposed procedures. Because of high sensitivity, these procedures would be suitable for the determination of befunolol and M1 in samples of low concentrations.

6.1.4.7 Radioimmunoassay for Carazolol

Carazolol, 1-(4-carbazolyloxy)-3-isopropylamino-2-propanol, is a beta-blocker effective for the prevention of stress-induced tachycardia in pigs. Administration of carazolol during the transportation of pigs to the slaughterhouses is not allowed. Little is known about the presence of residual carazolol in pigs because no efficient procedure is available for the routine detection of the drug. In order to control the illegal use of carazolol in the slaughterhouses, Rattenberger et al. have developed a radioimmunoassay for the drug in the blood and urine of pigs (ref. 13).

Preparation of the immunogen and the antisera. A derivative of carazolol, N-[3-(carbazolyl-4-oxy)-2-hydroxy-propyl]-alpha-amino-isobutyric acid, was coupled to BSA. The immunogen was injected into rabbits subcutaneously and intramuscularly to obtain the antisera.

Procedures for assay. An ^{125}I-labeled carazolol derivative, 15-(4'-amino-3'-iodobenzyl)-carazolol, with a specific activity of about 2000 Ci/mmol was used as the label. Phosphate buffer (0.2 M, pH 7.2) was used as the working solution. To a glass test tube containing the label (20,000 dpm) and 100 μl of the standard or dry extract of the HPLC dissolved in the working solution was added 500 μl of the diluted antiserum in phosphate buffer containing BSA. The contents of the tubes were incubated at 37°C for 15 min and then in ice-water bath for 1 hr. The label bound to the antibody was separated from the free label by the DCC method.

Specificity. Since the metabolism of carazolol in pigs is not known, it is impossible to determine the specificity of the assay. Therefore, biological samples were subjected to extraction into ether followed by purification with HPLC before radioimmunoassay in order to maximally guarantee the specificity of the assay.

Sensitivity. The lower limit of the sensitivity was 0.1-0.2 ppb and 0.1-0.4 ppb for plasma and urine samples, respectively.

Reproducibility. Average intra-assay covariation of carazolol in plasma and urine samples was 8%, and inter-assay covariations in plasma and urine were 23 and 16%, respectively.

Application. After intramuscular administration of carazolol (1.5 mg) to pigs weighing about 90 kg, plasma and urine samples were collected for 48 hr. After extraction and HPLC procedures, determination of carazolol was performed by radioimmunoassay. In plasma and urine, it was found that a detectable amount of carazolol was present for up to 8 and 24 hr after the administration, respectively.

6.1.5 APPLICATION OF IMMUNOASSAYS FOR THE DETERMINATION OF BETA-BLOCKERS IN BIOLOGICAL MATERIALS

Determination of the tissue content and plasma concentrations of dl- and l-propranolol with a stereospecific radioimmunoassay in mice, rabbits and hyperthyroid patients after the administration of the racemic drug is briefly reviewed in this section (ref. 15-17). These studies were made possible by the development of the stereospecific radioimmunoassay for propranolol isomers (ref. 2).

6.1.5.1 Heart Content of Propranolol Isomers in Mice (ref. 15)

After intravenous injection of 16 mg/kg dl-propranolol, groups of 5 or more mice were killed at the designated times. The heart was removed rapidly and homogenized in 0.05 N HCl. The supernatant was obtained by centrifugation of the homogenate at 5,000 x g for 15 min and diluted with phosphate-buffered saline. A portion of the diluted supernatant was transferred to a tube containing standard assay reagents and determined for dl- and l-propranolol contents by the stereospecific radioimmunoassay (ref. 2). The values for d-propranolol content were calculated by subtracting the values for the l-isomer from the results obtained for dl-propranolol. In the first 15 min, twice as much l-propranolol was found in the heart as the d-isomer (Fig. 6.1.6). The data indicate a preferential initial uptake of l-propranolol to the heart over the d-isomer.
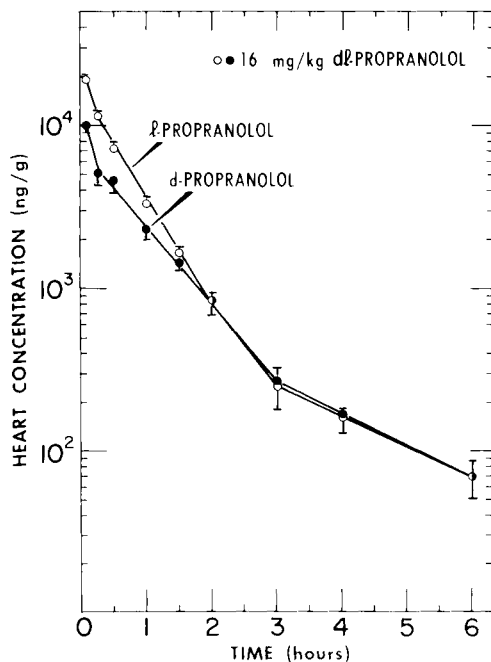
Fig. 6.1.6. Heart concentrations of propranolol isomers after intravenous injection of 16 mg/kg dl-propranolol in mice (results given for d- and l-isomers separately). Adapted from A. Levy et al. (ref. 15) with permission of the European Journal of Pharmacology.

6.1.5.2 Plasma Concentrations of Propranolol Isomers in Rabbits (ref. 16)

It has been suggested that the half-life of l-propranolol is longer than that of the d-isomer after intravenous injection of the racemic drug in the rat (ref. 2). Thus, studies on the pharmacokinetics of dl- and l-propranolol after intravenous injection of 200, 400 and 800 μg/kg of the racemic drug in rabbits were performed using stereospecific antisera with radioimmunoassay procedures. The l-isomer had a longer plasma half-life than dl-propranolol at each dose during the beta-phase (Fig. 6.1.7). At steady state, plasma concentrations of both dl- and l-propranolol correlated well with beta adrenoceptor blocking activity (r = 0.913 for dl-propranolol and r = 0.939 for the l-isomer). The results indicate that plasma concentrations of dl- and l-propranolol are good parameters for beta blocking activity.
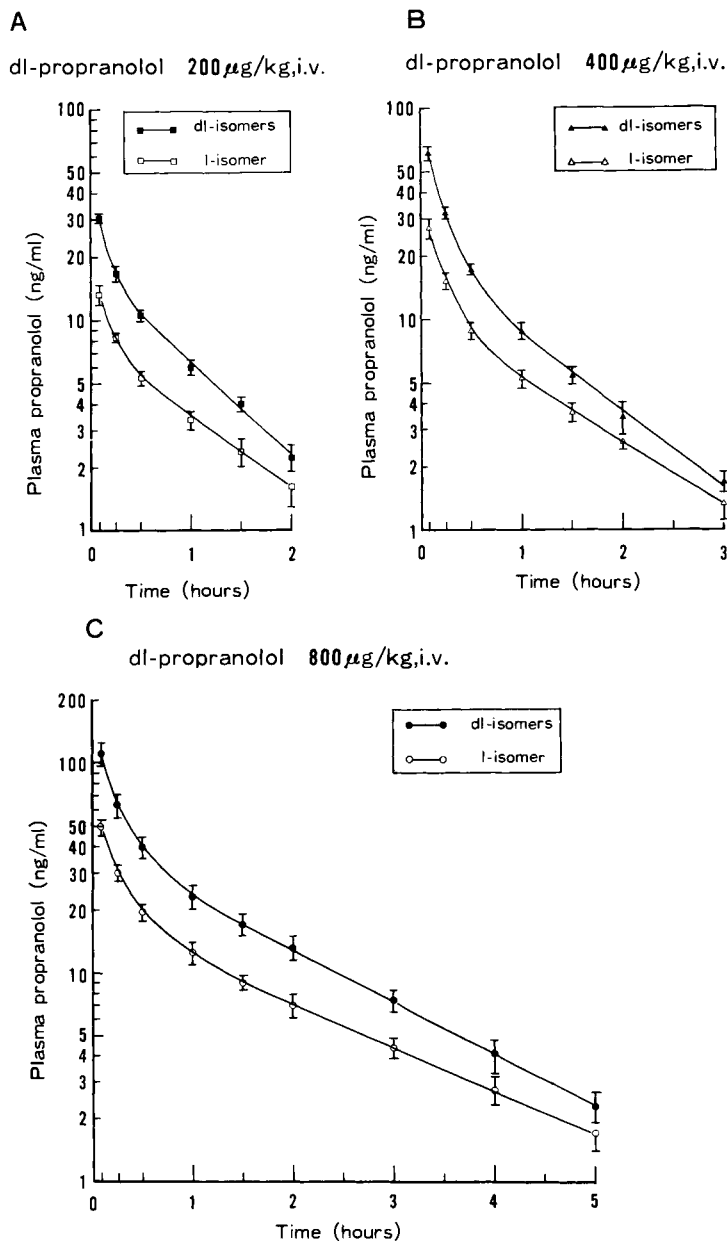
Fig. 6.1.7.   Semilogarithmic plot of propranolol isomers concentration-time curves after 200 (A), 400 (B) and 800 (C) μg/kg intravenous injection of dl-propranolol in 6 rabbits. Reprinted from K. Kawashima and H. Ishikawa (ref. 16) with permission of Williams & Wilkins.

6.1.5.3 Plasma Concentrations of Propranolol Isomers in Hyper-
thyroid Patients (ref. 17)

The pharmacokinetics of propranolol stereoisomers were deter-
mined after a single oral administration of the racemic drug in
seven hyperthyroid patients before and after antithyroid drug
therapy. The patients were given 20 mg x 2 or 3 oral dose of
dl-propranolol hydrochloride tablets (average dose 1.23 ± 0.04
mg/kg), and blood samples were obtained at the designated times.
Plasma concentrations of dl- and l-propranolol were determined by
stereospecific radioimmunoassay (ref. 2). The plasma concentra-
tion-time curves for both dl- and l-propranolol were monophasic,
indicating that the one-compartment open model is applicable for
the analysis of the data. No significant difference in half-life
between dl- and l-propranolol was observed before and after
antithyroid drug therapy (Fig. 6.1.8). However, plasma clearance
values for l-propranolol were smaller than those for dl-proprano-
lol, and the difference was statistically significant after
antithyroid drug therapy.
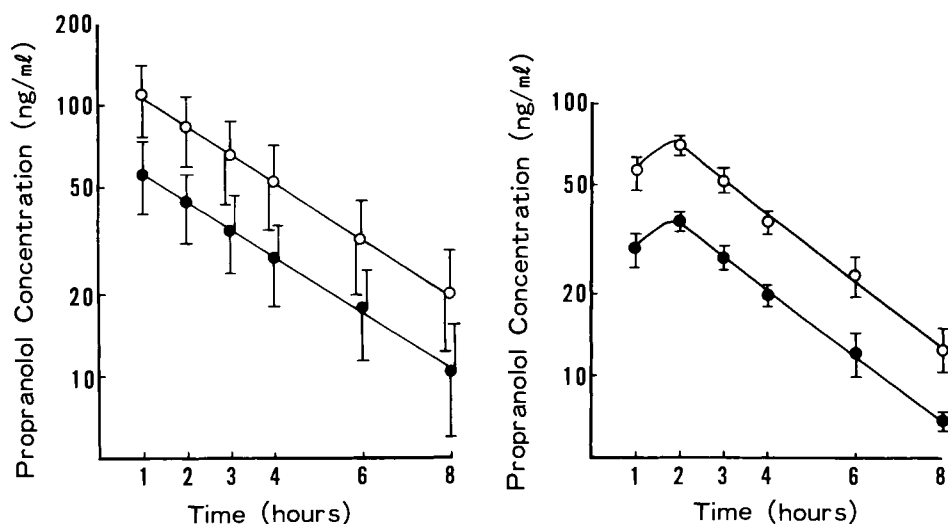
Fig. 6.1.8. Semilogarithmic plot of concentration-time curves of
propranolol isomers after a single oral administration of 40-60 mg
dl-propranolol (average dosage, 1.23 ± 0.04 mg/kg) in 7 hyper-
thyroid patients before (a) and after (b) antithyroid drug thera-
py. Open circles, dl-propranolol; closed circles, l-propranolol.
Reproduced from K. Tawara et al. (ref. 17) with permission of
Springer-Verlag.

These findings indicate that there are some differences in pharmacokinetics between the stereoisomers of propranolol. These differences appear to depend, at least in part, on the dose, the route of administration, and the species used for the study.

6.1.6 ADVANTAGES AND LIMITATIONS OF IMMUNOLOGICAL METHODS

The sensitivity of an immunoassay largely depends on the affinity of the antibody and the specific activity of the label. In general, radioimmunoassays have higher sensitivities than other immunoassays which utilize the enzyme or fluorescent labels. In the case of an enzyme immunoassay, the heterogenous type of immunoassay which requires the separation of the antibody-bound label from the free label is more sensitive than the homogeneous type of immunoassay which does not require any separation steps. Most immunoassays for beta-blockers discussed above are sensitive enough to determine plasma concentrations of drugs in clinical samples of a small volume (10 to 100 µl) without requiring prior extraction and concentration steps. However, in the case of the enzyme immunoassay for befunolol, an acetone extraction step was required because of the presence of interfering substances in the plasma (ref. 12).

Since the antibody can discriminate between the stereoisomers, it is possible by immunoassay using stereospecific antiserum to selectively determine only one isomer of a beta-blocker in the presence of the other isomer. The stereospecific radioimmunoassay for propranolol isomers (ref. 2) is one of the examples of this technique.

The specificity of an immunoassay is closely related to the cross-reactivity of the antiserum with the metabolites and the interaction with the substances present in the samples. Comparison of the results of the immunoassay with those obtained by other established procedures would be useful for the evaluation of the method. Results from the fluoroimmunoassay for propranolol in patient's serum or plasma samples using the antiserum raised against propranolol hemisuccinate-BSA conjugates correlated well with the data from the fluorometric determination (ref. 5), while values obtained by radioimmunoassay for propranolol in human plasma using the antiserum raised against the ring-conjugted immunogen was about 10-fold higher than those obtained by GC/MS

(ref. 4). Thus, the interference with the assay by the metabolites can be modified by the preparation of a proper immunogen, because it is now well-known that antiserum shows its specificity for the part of the hapten molecule furthest from its site of conjugation to the carrier protein (ref. 14). As for the immuno-assay for propranolol, the antiserum raised against the side chain-conjugated immunogen appears to be more suitable than that raised against the ring-conjugated immunogen. Although it may affect the simplicity of operation and high throughput of an immunoassay, addition of extraction steps before the assay has been found to improve the specificity of the assay (ref. 4 and 12).

It is reported that the precision and accuracy of most radio-immunoassays do not approach those of a good chemical method but are comparable to those of chromatographic procedures and better than those of biological ones (ref. 1). In most immunoassay procedures for beta-blockers, the coefficient of variation of inter- and intra-assay covariances has been reported to be within 10%. However, in some cases, the coefficient of variation reached to around 15% (ref. 3, 6 and 12). Thus, it would be advisable to avoid the assay at the edges of the standard curve.

The development of an immunoassay procedure requires a certain period of time and a specially equipped laboratory in the case of radioimmunoassays. However, once it is established, it is less costly and simple to perform. Thus, high throughput can be expected. Furthermore, because of its sensitivity, only a small volume of samples is required. The specificity of an immunoassay sometimes is a matter of dispute. If the specificity of an immunoassay is assured by comparison of the results between the immunoassay and other established methods, it is possible to directly apply plasma or serum samples to the assay. Even when an extraction procedure is required prior to the assay, an immuno-assay would be still attractive in many instances.

REFERENCES

1 V.P. Butler, Jr., Pharmacol. Rev., 29 (1978) 103-184.
2 K. Kawashima, A. Levy and S. Spector, J. Pharmacol, Exp.
   Ther., 196 (1976) 517-523.
3 G.P. Mould, J. Clough, B.A. Morris, G. Stout and V. Marks,
   Biopharm. Drug Dispos., 2 (1981) 49-57.

4   T.D. Eller, D.R. Knapp and T. Walle, Anal. Chem., 55 (1983)
    1572-1575.
5   M.H.H. Al-Hakiem, G.W. White, D.S. Smith and J. Landon, Ther.
    Drug Monit., 3 (1981) 159-165.
6   K. Chegwidden, M.R. Pirio, P. Singh, J.B. Gushaw, J.G. Miller
    and R.S. Schneider, Clin. Chem., 24 (1978) 1056.
7   B. Gourmel, J. Fiet, R.F. Collins, J.M. Villette and C. Dreux,
    Clin. Chim. Acta, 108 (1980) 229-237.
8   K. Kawashima, J. Pharmacobio-Dyn., 4 (1981) 534-540.
9   S.-Y. Chu, S.M. Vega, A. Ali and L.T. Sennello, J. Pharm.
    Sci., 70 (1981) 990-994.
10 B. Gourmel, J. Fiet, R.F. Collins, J.M. Villette, P. Pissa and
    C. Dreux, Clin. Chim. Acta, 115 (1981) 229-234.
11 K. Kawashima and A. Nagakura, Japan. J. Pharmacol. 33 (1983)
    141P.
12 S. Sato and I. Yamamoto, J. Immunoassay, 4 (1983) 351-371.
13 Von E. Rattenberger, P. Matzke and J. Neudegger, Arch.
    Lebensmittelhygiene, 36 (1985) 85-87.
14 K. Landsteiner, Specificity of Serological Reactions, Harvard
    Univ. Press, Chambridge, Mass., 1945.
15 A. Levy, S.H. Ngai, A.D. Finck, K. Kawashima and S. Spector,
    Eur. J. Pharmacol., 40 (1976) 93-100.
16 K. Kawashima and H. Ishikawa, J. Pharmacol. Exp. Ther., 213
    (1980) 628-631.
17 K. Tawara, K. Kawashima, H. Ishikawa, K. Yamamoto, K. Saito,
    A. Ebihara and S. Yoshida, Eur. J. Clin. Pharmacol., 19
    (1981) 197-203.
18 R. Edwards, Immunoassay: An Introduction, Heineman, London,
    1985.
19 J. Landon and A.C. Moffat, Analyst 101 (1976) 225-243.
20 W.M. Hunter and F.C. Greenwood, Nature (London), 194 (1962)
    495-496.
21 R.S. Farr, J. Infect. Dis., 103 (1958) 239-262.
22 W.L. Nelson and R.B. Walker, Res. Commun. Chem. Pathol.
    Pharmacol., 22 (1978) 435-445.
23 R.S. Kamel and J. Landon, Clin. Chem., 26 (1980) 1281-1286.
24 D.G. Shand, E.M. Nuckolls and J.A. Oates, Clin. Pharmacol.
    Ther., 11 (1970) 112-120.
25 R.F. Collins, Nouv. Presse Med., 4 (1975) 3223-3228.
26 A. Roux and B. Flovat, J. Chromatogr., 106 (1978) 327-332.
27 T. Nambara, S. Akiyama and M. Kurata, Yakugaku Zasshi, 93
    (1973) 439-441.
28 M. Schafer and E. Mutschler, J. Chromatogr., 164 (1979)
    247-252.
29 K. Kawashima, Y. Miwa, K. Fujimoto, J. Matsumoto, M. Kimura
    and A. Nagakura, Japan. J. Pharmacol., 38 (1985) 259-265.

Chapter 6.2

RADIORECEPTOR ASSAY OF ß-BLOCKERS (RRA)

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6.2.1 INTRODUCTION

The measurement of drug concentrations in biological fluids (e.g. plasma, urine, saliva) is of essential clinical value, if the effects or side-effects can be related with such data. Furthermore, one may then be able to predict the necessary doses and dosage intervals from pharmacokinetic measurements and thus achieve maximum of therapeutic effects at a good benefit/risk ratio (refs. 1-5). The principles of the RRA of plasma concentrations in relation with the effects in vivo are shown in Fig. 6.2.1. Three different "compartments" are shown in this graph: The assay tube in the laboratory, the plasma compartment and the effect compartment in vivo.
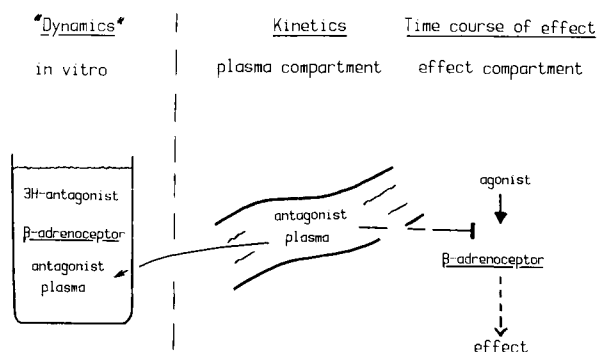
Fig. 6.2.1. Relationship between the in vitro RRA and the in vivo plasma and effect compartment.

The rationale behind the RRA of ß-blockers (refs. 6-9) is the well-quantified interaction of these competitive antagonists with ß-adrenoceptors independent from the investigational method used: Effect measurements in man (refs. 10-18) or in intact animals, studies in isolated tissues, cells, cel-

lular membranes or purified and cloned ß-adrenoceptors (refs. 19-23). The respective common characteristic constant of each of the drugs is the dissociation constant (the K_d- or K_i-value; refs. 23-26). Thus, in Fig.6.2.1 ß-adrenoceptors present in man and in the assay tube are shown in the same style. The proof for this assumption will be substantiated further below (6.2.4).

Due to the present topic, the first part (6.2.2) of the paper emphasizes methodological problems (radioligands, ß-adrenoceptor preparation, treatment of biological samples, incubation, data evaluation, sensitivity of the RRA). In the second part (6.2.3), the validation of the RRA by physico-chemical detection methods of racemates or enatiomers and the role of active metabolites present in the samples is described. Finally, in the third part (6.2.4), the results derived from the RRA are compared with effect measurements in man. This last part deals with the crucial point of any detection method used in pharmacokinetic studies, i.e. to which extent can from the in vitro data be extrapolated to effects in man (refs. 1,3,18,27)?

6.2.2 METHODS
6.2.2.1 Radioligands

A large series of radiolabelled ß-blockers is commercially available from common sources (e.g. Amersham-Buchler, Braunschweig FRG; NEN, Dreieich FRG; refs. 6-9,28-30). If protein-free samples are investigated, lipophilic radioligands (with high protein binding) may be used without problems. As shown in Fig. 6.2.2 (left panel), receptor binding of the radioligand [3]H-dihydroalprenolol ([3]H-DHA) declines with increasing amounts of plasma included with the assay.
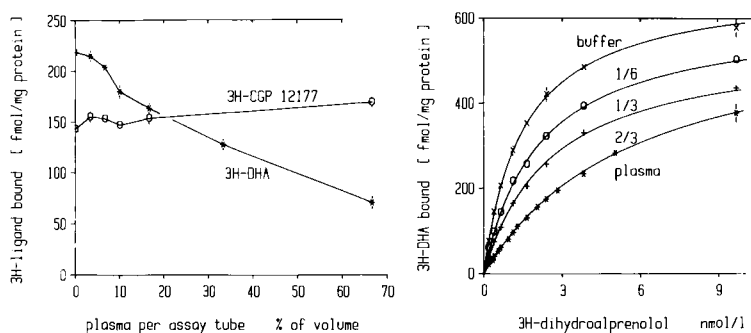
Fig. 6.2.2. Left: The influence of the addition of plasma on receptor binding of a lipophilic ([3]H-DHA) and a hydrophilic radioligand ([3]H-CGP 12177). Right: Saturation isotherms of [3]H-DHA in the presence of increasing amounts of plasma (Data taken from ref. 31).

The decline of receptor binding of ^{3}H-DHA is due to a reduction of the free radioligand concentration by its binding to the plasma proteins added (refs. 6,7). In the saturation isotherms shown in Fig. 6.2.2, the rightward shift of the apparent K_d-value of the radioligand due to the diminished free radioligand concentrations with increasing amounts of plasma becomes apparent.

To be independent from plasma protein binding of the radioligand, we use the hydrophilic radioligand (-)^{3}H-CGP 12177 (ref. 32). As can be read from Fig. 6.2.2 (left panel), receptor binding of this radioligand is not affected by the inclusion of plasma protein in the incubation. Therefore, ^{3}H-CGP 12177 can be suggested as the most appropriate for the purposes of the ß-blokker RRA. The K_d-values of this ligand in different membrane preparations (e.g. from rats: cerebral cortex, heart, salivary glands, reticulocytes) are 0.4 to 0.8 nmol/l and the ligand is thus rather non-selective in terms of ß-adrenoceptor subtypes (refs. 33,34). The specific activity is 30 to 50 Ci / mmol and thus preparations with ß-adrenoceptor concentrations above 50 fmol/mg of protein are suitable as biological material.

6.2.2.2 ß-Adrenoceptor preparation

Numerous studies describe the necessary prerequisites for the characterization of ß-adrenoceptors by radioligand binding studies (refs. 6,7,23,28). For the purposes of the RRA, preparations containing ß-adrenoceptors above 100 fmol/mg of protein should be used to get a good signal. Usually, crude membrane preparations from ß-adrenoceptors containing tissues are sufficient due to the low non-specific binding of the hydrophilic radioligand ^{3}H-CGP 12177. We use rat salivary gland and reticulocyte membranes, because either of them contain only one ß-adrenoceptor subtype at densities above 200 fmol/mg of protein (refs. 35,36). By this, subtype-selective ß-blockers can also be detected as described below for atenolol, metoprolol and bisoprolol.

The experimental approach to detect the ß-adrenoceptor subtype present in the membrane preparation used, is exemplified in Fig. 6.2.3. Competition isotherms of subtype-selective ligands (bisoprolol and ICI 118,551) and of propranolol at rat salivary gland membranes are depicted. One can read from this figure, that parallel curves are obtained, indicating the presence of only one subtype in this preparation. Since bisoprolol ($ß_1$-selective) is about 100-fold more potent than ICI 118,551 ($ß_2$-selective), only $ß_1$-adrenoceptors are labelled by ^{3}H-CGP 12177. With rat reticulocyte membranes the inverse potency of ICI 118,551 / bisoprolol was observed. This preparation thus contains only $ß_2$-adrenoceptors (ref. 34). If both ß-adrenoceptor subtypes are present in a preparation, one may add a selective ligand at concentrations sufficient to block one of the subtypes (ref. 37) and thus assay concentra-

tions of selective antagonists. However, this procedure is quite sophistica-
ted and makes data evaluation from the RRA rather difficult.
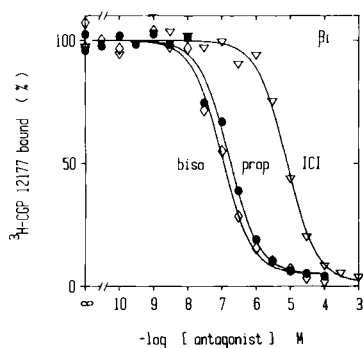
Fig. 6.2.3. Competition isotherms of various antagonists at rat salivary
gland ß-adrenoceptors. Antagonists with different $ß_1/ß_2$-selectivity profiles
were used: (+)-ICI 118,551 (1/300), (+)-propranolol (1/2), (+)-bisoprolol
(70/1).

6.2.2.3 Preparation of biological samples / plasma protein binding

Different plasma protein binding of parent drugs, active metabolites or
enantiomers can make pharmacokinetic measurements rather insufficient for a
comparison with effect data (refs. 27,38,39). Results obtained with the high-
ly lipophilic penbutolol (ref. 40) illustrate this aspect. In Fig. 6.2.4 the
effect of different plasma protein binding on the apparent potency of penbu-
tolol in comparison to the hydrophilic carteolol is shown. Both drugs are si-
milar in their potency to occupy ß-adrenoceptors in buffer medium. K_d-values
in the nanomolar range are observed.
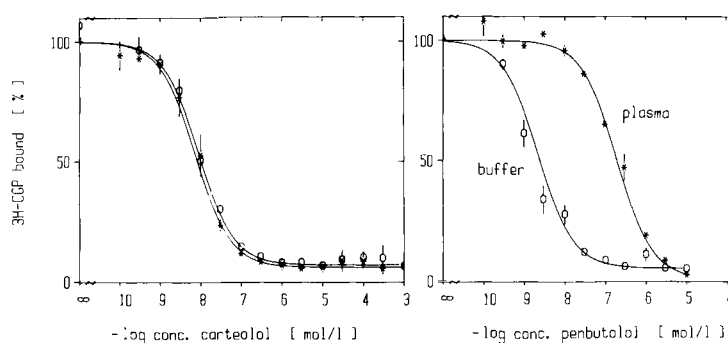
Fig. 6.2.4. Competition isotherms of penbutolol and carteolol at rat reticu-
locyte membranes without (o) and with (*)inclusion of plasma in the assay.

However, with the inclusion of plasma in the incubation medium, penbutolol looses almost two decades of potency in contrast to carteolol, which is not affected to a relevant extent.

High protein binding of penbutolol, and thus a reduced free concentration in the plasma medium, is responsible for the apparent loss of potency in plasma medium. Extraction of drug from plasma sampled after penbutolol administration will thus give erroneous result with respect to the ß-blockade expected to occur in man (ref. 41). It is worth to mention, that active metabolites of penbutolol (e.g. 4-OH-penbutolol) show substantially less protein binding than the parent drug (ref. 42). This makes a prediction of ß-blockade from a chemical detection of parent drug and/or metabolites very complicated, if not impossible. The respective results from the RRA of plasma sampled after administration of penbutolol in comparison to effect measurements in man are shown in Fig. 6.2.11 and discussed in the respective section (6.2.4.1).

To avoid the bias encountered with the extraction of plasma samples, we prefer the use of native samples. After obtaining venous blood, plasma is separated by centrifugation and kept frozen (-20°C) until use. Samples are only thawed once for the unprocessed use in the RRA. By this, free drug concentrations are assayed with the RRA and a good agreement between the in vitro ß-blockade detected by the RRA and the effects in man is obtained (see section 6.2.4).

It should be stressed, that calibration curves used to evaluate the RRA measurements (see section 6.2.2.5) should be run in the presence of plasma obtained from a drug-free period of the individual from whom samples with ß-blocker present are measured. Urine and saliva samples can be assayed in the same manner as plasma samples. However, care should be taken to obtain calibration curves in the presence of urine or saliva from a drug-free period.

6.2.2.4 Incubation and separation of free from bound radioligand

The sensitivity of the RRA in part depends on the amount of sample included in the incubation volume (ref. 6,7,31). We use 200 µl of plasma samples in the incubation. 50 µl of the suspension containing the ß-adrenoceptor preparation, 20 µl of radioligand and 30 µl of buffer or unlabelled ligand are added. Thus, the original plasma sample is only diluted by 1/3. The total incubation volume of 300 µl is filtered through glass fibre filters after the equilibration period (60 to 90 minutes at 25°C) to separate free from bound radioligand.

Filtration devices are available from common laboratory equipment faci-
lities and offered by several specialized companies (e.g. Schleicher-Schüll;
Millipore; BRANDEL; Skatron; refs. 28-30). A tremedous amount of unpleasant
and time-consuming laboratory work can be saved by the use of multifold fil-
tration devices offered by these manufacturers.

The choice of filter materials depends on the ß-adrenoceptor preparation
used. Millipore filters (AP15 or AP20) and Whatman GFB or GFC filters are the
most appropriate and commonly used (refs. 28-31,43).

6.2.2.5 Data evaluation

Several evaluation methods of the RRA (refs. 28-31) have been adopted for use
with computer facilities (e.g. LIGAND for IBM-PC available from Elsevier-Bio-
soft). We have developed a package for the Hewlett-Packard series 200 desk
top calculators (GIP =Gießen Iteration Procedure) for non-linear, weighted,
least squares curve fitting. Copies are available from the author (refs. 33,
35). In the curve-fitting procedures describing the interaction of radioli-
gand and/or unlabelled antagonist with ß-adrenoceptors the following equation
according to the law of mass action is used independent from the software
package:

$$B_{Li} = (B_{max} \times L) / (L + K_d \times (1 + i/K_i)) \quad + \quad nsb \times L \qquad (6.2.1)$$

In this equation B_{Li} is radioligand bound at concentration L and concentra-
tion i of the antagonist. B_{max} is the binding capacity and K_d the dissoci-
ation constant of the radioligand and nsb its non-specific binding. K_i is the
dissociation constant of the antagonist.
If competition isotherms with unlabelled antagonist (see Fig. 6.2.3 or 6.2.4)
are used as calibration curves, equation 6.2.1 can be simplified to

$$B_{Li} = B_0 / (1 + (i/IC_{50})) \quad + \quad nsb \qquad (6.2.2)$$

In this equation B_{Li} is radioligand bound at concentration i of the antago-
nist, B_0 the amount bound at i=0 and IC_{50} the half-maximal inhibitory concen-
tration. Unknown concentrations can be read (or computed) from such calibra-
tion curves and may be given in concentrations equivalent to the respective
ß-blocker used for the calibration. The standard evaluation of the RRA data
by the use of calibration curves with unlabelled ß-blockers should give data
on the identity line between the RRA and a chemical detection method (see
6.2.3; ref. 44). However, no judgement concerning the pharmacologic activity

can be made from such data. Furthermore, with active metabolites and enantio-
mers, this procedure will yield biased results (see 6.2.3.1 and 6.2.3.2).

Therefore, we suggest an evaluation method of the RRA data which gives the
results in terms of pharmacologic activity present in the samples: Either in
fraction of ß-adrenoceptors occupied by the antagonist present or concentra-
tions in multiples of the dissociation constant (i/K_i). For this purpose a
saturation isotherm of the radioligand (with drug-free human plasma) is used
as the calibration curve. From this data B_{max}, K_d and nsb of the system are
calculated. In a parallel assay, plasma obtained after serum administration
is incubated with a fixed concentration of radioligand and the amount of
receptor binding (B_{Li}) is determined. Equation (6.2.1) can be solved for i/K_i
and unknown antagonist concentrations in the samples can then be calculated
from the data:

$$i/K_i = ((B_{max} \times L) / (B_{Li} - L \times nsb) - L) \times 1/K_d) - 1 \qquad (6.2.3)$$

The receptor occupancy can be calculated from

$$\text{Fractional occupancy} = 1 - (1 / (1 + i/K_i)) \qquad (6.2.4).$$

Results derived from this evaluation method are exemplified in Fig. 6.2.5.
From the abscissa (RRA), one can judge the relative β_1-adrenoceptor potency
of the doses administered: atenolol 200 mg / bisoprolol 100 mg / propranolol
240 mg orally = 1 / 5 / 8.

RRA: log conc. antagonist [i/K$_{i1}$]
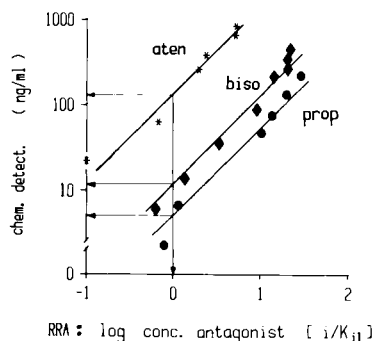
Fig. 6.2.5. Plasma concentrations after oral administration of propranolol
240 mg, atenolol 200 mg and bisoprolol 100 mg. 3 to 84 hours after a single
dose, plasma concentrations were detected by the RRA with rat salivary gland
membranes (β_1-subtype; given in multiples of the K_i-value=i/K_i) and by HPLC
(racemic drug). (Data taken from ref. 42).

Furthermore, one may relate plasma concentration detected by the HPLC-method (ordinate) to the potency at β_1-adrenoceptors. The plasma concentrations equivalent to the K_i-value (and hence a 50% receptor occupancy) were: 6 ng/ml for propranolol, 10 ng/ml for bisoprolol and 150 ng/ml for atenolol. The relevance of this evaluation method for pharmacodynamic studies is outlined in section 6.2.4.

6.2.2.6 Sensitivity and detection limits

One crucial point in any of the assay methods described in this volume is the sensitivity of detection. Since the RRA employs β-adrenoceptors as the matrix for the detection, the sensitivity is related to the ratio of concentration/K_d-value. In other words, low concentrations of an antagonist with a low K_d-value (=high affinity) can be detected and vice versa. By the RRA, the inhibition of radioligand binding between 10% and 90% of specific binding can be quantified with good precision (see Fig. 6.2.3). The concentration of radioligand used in the assay determines, which antagonist concentration inhibits radioligand binding by 10% to 90%. With increasing concentrations of the radiolabel, the sensitivity is decreased. Table 6.2.1 describes this relation.

TABLE 6.2.1

Sensitivity of the RRA and detection limits depending on the concentration of the radioligand. Radioligand concentrations are given in multiples of the K_d-value (L/K_d). The sensitivity is given in % of β-adrenoceptor occupancy in the assay by the concentration of unknown antagonist inhibiting radioligand binding by 10 %. The detection limits of the assay (between 10 % and 90 % inhibition = IC_{10} and IC_{90}) are calculated in multiples of the K_i-value of the unknown antagonist. It is assumed that 2/3 of the assay volume consist of the native sample. Equation 6.2.1 was used for the calculations.

radioligand concentration $\frac{}{}$ (L/K_d)	0.1	0.3	0.5	1	3	5	10
sensitivity (% occupancy)	15	17	20	25	40	50	64
IC_{10} (multiples of K_i)	0.18	0.21	0.25	0.33	0.67	1.00	1.79
IC_{90} (multiples of K_i)	18	21	25	33	67	100	179

The relevance of a these detection limits must be discussed in terms of effects in man. In general, the detection limits of any methods can be classified with a simple calculation: ß-blockers (like many other drugs) are administered orally in µmol to mmol doses with oral absorption rates above 10%. Thus, 10^{17} to 10^{20} molecules must finally be eliminated from the body. Taking the distribution volume into account, about 15 decades of drug concentrations

(10^{15} to 10 molecules / ml of plasma) are observable in the plasma compart-
ment during drug elimination. However, a detectable ß-blockade in man is only
seen within the first two decades of the ß-blocker elimination time course
(refs. 12-14,33,45,46) or within two decades of plasma concentrations in
dose-response curves (Fig. 6.2.6, right panel; refs. 10,11,15-17). Thus, for
comparison with clinical effect data, two decades of sensitivity below the
maximum plasma concentrations should usually be sufficient. As shown in the
comparison with effect data (6.2.4), indeed the detection limits of the RRA
are congruent with the detection limits of ß-blocker effects in man.

6.2.3 VALIDATION OF THE RRA USING CHEMICAL DETECTION METHODS

6.2.3.1 Racemates / enantiomers

With the usual chemical detection methods, plasma concentrations of race-
mate is detected after the administration of racemic ß-blockers. The RRA re-
sponds mainly to the (-)-enantiomers due to a stereoselectivity ratio between
(-) and (+)-enantiomers of about 2 decades. Plasma concentrations detected
from the RRA can be read from calibration curves with the respective race-
mate. The data are in agreement with results from the chemical detection if
active metabolites are absent. This situation is exemplified for (+)-propra-
nolol in Fig. 6.2.6 (left panel).
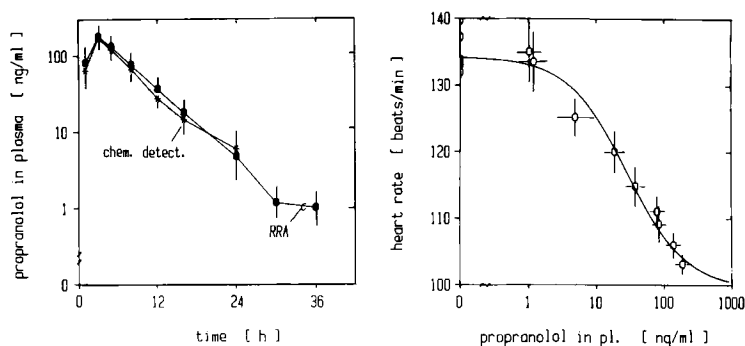
Fig. 6.2.6. Left: Plasma concentration kinetics of (+)-propranolol after a
single oral dose of 240 mg dose assayed by the RRA and a chemical detection
method. Right: Concentration-response relation at exercise heart rate (n=6
volunteers; data taken from refs. 31,33).

Several reports on stereoselective metabolism and eliminiation of ß-blockers
in man (refs. 48-50) or animals (ref. 51) make a stereospecific chemical de-
tection worthwhile. Fig. 6.2.7 shows a comparison between results derived
from the RRA and from a stereospecific HPLC-detection of (-)- and (+)-meto-
prolol after oral administration of 100 mg of racemate to healthy volunteers.

A good agreement is seen between the detection of the (-)-enantiomer and the RRA. However, the calculation of RRA data in equivalents of the (+)-enantiomer overestimates the respective plasma concentrations. Several conclusions can be drawn from these results: As outlined in the section on data evaluation of the RRA (6.2.2.5), concentration calculated from calibration curves with unlabeled antagonist only yields results in "concentration equivalents" relative to the respective calibration curve. If the "false" drug is used for calibration, the result will be biased in comparison to the "true" detection of antagonist by the chemical detection method. On the other hand, one can check this way, which enantiomer (or metabolite) is effective at ß-adrenoceptors after administration. Furthermore, a validation of the stereospecific chemical detection method is thus possible.
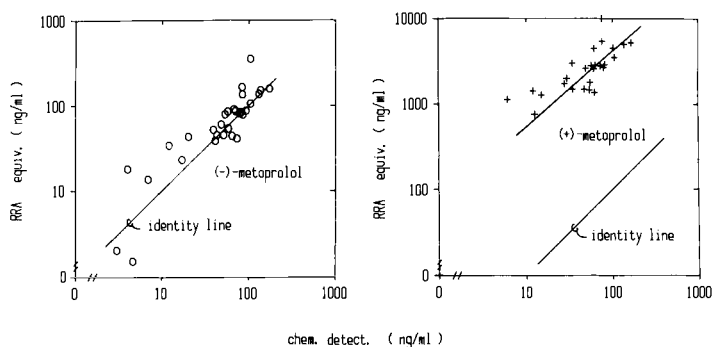
Fig. 6.2.7. Plasma concentrations 1 to 48 hours after oral administration of 100 mg of (+)-metoprolol to 5 healthy volunteers as detected by the RRA and by an enantiospecific HPLC-method. Left: (-)-metoprolol. Right: (+)-metoprolol (Data taken from ref. 47).

6.2.3.2 Active metabolites

Metabolites are formed from almost any of the ß-blockers in current therapeutic use (refs. 41,52). The question is, how much of these metabolites (relative to their potency) appears in the central compartment, i.e., whether metabolites contribute to the ß-blocking effects or not. A comparison between data from the RRA and chemical detection can help to solve this problem.

As exemplified for carteolol in Fig. 6.2.8, an increasing deviation between plasma concentrations detected from the RRA and from chemical detection becomes apparent during antagonist elimination. The difference between the two detection methods is due to an active metabolite, since the detection of parent drug in plasma samples spiked with carteolol only yielded a good agreement between the two detection methods. Antagonist concentrations in plasma stay above the K_i-value within the time interval covered by plasma

sampling. The ß-blockade in man measured by the reduction of exercise tachycardia was apparent until 96 h after administration of carteolol and is thus obviously due to the active metabolite calculated from the differences between the results from the two detection methods (ref. 31). Thus, parallel measurements by the two independent methods can be helpful to understand the effect kinetics of a ß-blocker with active metabolites.
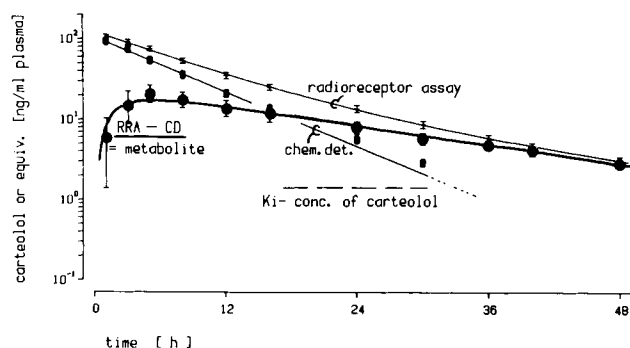
Fig. 6.2.8. Plasma concentration kinetics after oral administration of 30 mg of (+)-carteolol to 6 healthy volunteers measured by the RRA and a chemical detection method. The difference between the results from the two detection methods was calulated as active metabolite. (Data taken from ref. 31).

6.2.4 VALIDATION OF THE RRA USING EFFECT MEASUREMENTS IN MAN

The relevance of concentration measurements in biological fluids can be tested by a comparison with the pharmacologic effects predicted from the in vitro data. In studies with rats, Sriwatankul and Nahorski (ref. 43) have used an elegant approach to relate ß-adrenoceptor occupancy in different tissues with plasma concentrations: They administered different doses of propranolol or atenolol and detected the amount of ß-adrenoceptor occupancy in various tissues in comparison to plasma concentrations. A direct relationship between plasma concentrations and receptor occupancy was shown in these studies. In man, a parallel decline between the right shift of an isoprenaline dose-response at the heart rate and plasma concentrations of propranolol was reported by DeLeve et al. (ref. 12). We have shown in earlier studies, that plasma concentrations can be used to predict the effect kinetics of propranolol and atenolol, if the K_i-values detected from the RRA are used as a link between pharmacokinetics and pharmacodynamics (refs. 18,46). As will be shown in this section, the RRA can be used to predict ß-blockade in man.

This prediction is not only possible for stead-state administration (Fig.

6.2.9) but also during the elimination phase after a single oral dose (Figs. 6.2.10 and 6.2.11). In addition, the use of a β_1- and a β_2-adrenoceptor preparation in the RRA can relate the effects observed to the respective β-adrenoceptor subtype population (Fig. 6.2.12).

6.2.4.1 Non-selective antagonists

Bupranolol is an antagonist which is eliminated rapidly from plasma with a $t_{1/2}$ below 2 hours. In addition, most of a bupranolol dose administered oral- ly is extracted during the first pass (ref. 52). Due to the high lipophilici- ty, this β-blocker can be used for transdermal administration. With a trans- dermal system administered every 24 h, steady-state plasma concentrations can be achieved as can be read from the in vitro inhibition of receptor binding by plasma sampled during a 3 days administration (Fig. 6.2.9). The inhibition of exercise tachycardia reflects this in vitro measurement of β-adrenoceptor blockade.

time [h]
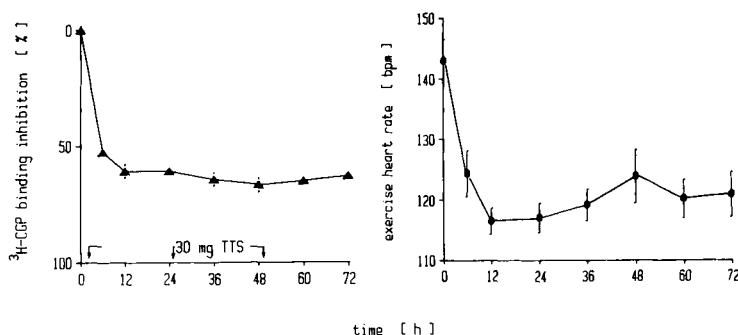
Fig. 6.2.9. Left: Inhibition of radioligand binding by antagonist present in plasma sampled during continuous administration of (+)-bupranolol by a trans- dermal therapeutic system (TTS). Right: Exercise heart rate observed in par- allel (n=6 volunteers; data taken from ref. 52).

A long duration of action of β-blockers despite a short elimination half-life has lead to many controversial discussions in literature (see refs. 18,46). E.g. propranolol is eliminated with a half-life of 3 to 4 hours. However, β-blockade in man after a single oral dose can be observed until 30 h after administration. By the use of the RRA in comparison to effect measurements, this apparent contradiction can be resolved (Fig. 6.2.10). The inhibition of receptor binding after propranolol administration by plasma samples assayed in the RRA and the inhibition of exercise tachycardia move in parallel. Thus, the duration of effect is dependent on the initial receptor occupancy -in other words: the dose in relation to the ED_{50}. A high dose will induce a

long-lasting effect (ref. 18,33).

Deep compartments of drug distribution or tight receptor binding of the
ß-blocker are not explanatory for the effect time course. Only the antagonist
concentration present in the plasma compartment is relevant for the
ß-blockade in man. The RRA of plasma samples provides clear evidence for this
interpretation (For a further discussion see ref. 33).
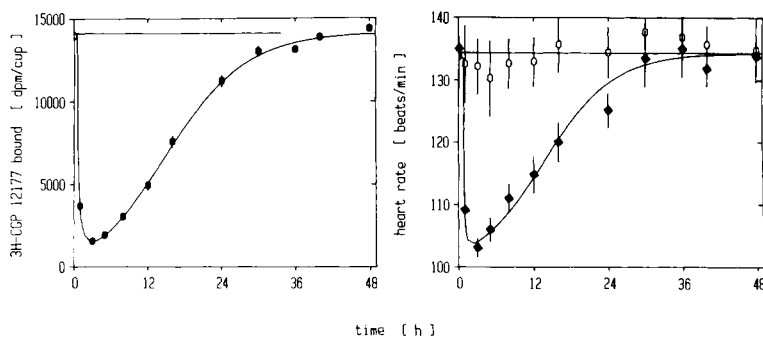
Fig. 6.2.10. Left: RRA of plasma samples after oral administration of 240 mg
of (+)-propranolol. Right: Exercise heart rate observed in parallel (-o-
=placebo). (n=6 volunteers; data taken from ref. 33)

Even for an antagonist with complex pharmacokinetics, the RRA can be used
to elucidate whether or not deep compartments beyond the plasma compartment
(ref. 54) contribute to the effects. Results obtained with the highly lipo-
philic penbutolol (refs. 41,42) can illustrate this situation (Fig.6.2.11).
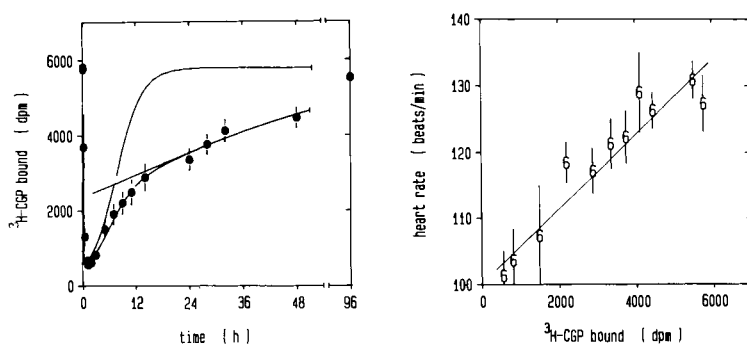
Fig. 6.2.11. Left: RRA of plasma samples after oral administration of 40 mg
of (-)-penbutolol. Right: Correlation between data obtained from the RRA and
the parallel measurements of heart rate (6 minutes of exercise; n=7 volun-
teers; data taken from ref. 53).

A biphasic decline of the inhibition of receptor binding by plasma sampled after administration of penbutolol is apparent. Despite this complex decline of in vitro ß-blockade, a linear relationship is observed between the results from the RRA and the inhibition of exercise tachycardia (Fig.6.2.11, right panel). Thus, one can conclude also for penbutolol, that free antagonist concentrations in plasma induce ß-blockade in man. It is worthwhile to note, that a comparison between the chemical detection of parent drug and the RRA data are indicative of active metabolites contributing to the overall effects observed after penbutolol (ref. 42,53).

6.2.4.2 β_1-selective antagonists

β_1-selective antagonists have been used widely in therapy (refs. 5,16). A subtype-selective RRA can relate the ß-blockade observed in man to the respective ß-adrenoceptor subtype. From the data depicted in Fig. 6.2.12, one can see, that after 200 mg of atenolol, maximum β_2-adrenoceptor occupancy detected by the RRA of plasma samples is below 30 %. After 12 hours, β_2-subtype occupancy is below the detection limit. For the β_1-subtype, receptor occupancy is detectable until 36 hours after drug administration. A comparison with the effect data derived from the inhibition of exercise tachycardia indicates that the β_1-subtype is involved with the effects in man with respect to the extent and duration of effects.

time (h)
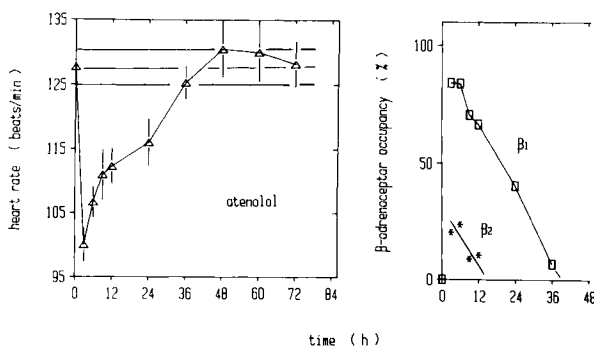
Fig. 6.2.12. Left: Exercise heart rate after oral administration of 200 mg of (+)-atenolol to n=6 volunteers. Right: ß-adrenoceptor subtype occupancy detected by the RRA of plasma samples with rat salivary gland (β_1) and rat reticulocyte membranes (β_2).

6.2.5 CONCLUSIONS

The RRA of ß-blockers is a very useful tool for the understanding of ß-blockade in man. However, this assay bears several pitfalls which can be

circumvented by the use of an appropriate radioligand, ß-adrenoceptor prepa-
ration and data evaluation method. In combination with chemical detection me-
thods, active metabolites and enantimers contributing to the effects can be
detected. The major advantage of the RRA is the direct relationship between
the in vitro results and the pharmacodynamics detected in man. This may even
be attributed to ß-adrenoceptor subtype driven effects. Thus, the RRA is a
useful tool to define equipotent doses of different ß-blockers and understand
the effect kinetics after drug administration in man. The claim for a rele-
vant detection method of drug concentrations with respect to effects (refs.
1,55), appears to be fulfilled by the RRA.

REFERENCES

1   H. Druckrey and K. Küpfmüller, Die Pharmazie 8 (Suppl. 1) (1949) 514-645.
2   N.H. Holford and L.B. Sheiner, Pharmac. Ther., 16 (1982) 143-166.
3   Z.H. Israili , Ann. Rev. Pharmacol. Toxicol., 19 (1979) 25-52.
4   G. Levy, Clin. Pharmacol. Ther., 7 (1966) 362-372.
5   A. Scriabine, Ann. Rev. Pharmacol. Toxicol., 19 (1979) 269-284.
6   D.B. Barnett, M. Batta, B. Davies and S.R. Nahorski, Eur. J. Clin. Phar-
    macol., 17 (1980) 349-354.
7   J.P. Bilezikian, D.E. Gammon, C.L. Rochester and D.G. Shand, Clin.
    Pharmacol. Ther. 26 (1979) 173-180.
8   R.B. Innis, D.B. Bylund and S.H. Snyder, Life Sci., 23 (1978) 2031-2038.
9   G.L. Rochester, D.E. Gammon, E. Shane and J.P. Bilezikian, Clin.
    Pharmacol. Ther., 28 (1980) 32-39.
10 C. Chidsey, M. Pine, L. Favrot, S. Smith, G. Leonetti, P. Morselli and A.
    Zanchetti, Postgraduate Med. J. 52 (Suppl. 4) (1976) 26-32.
11 D.J. Coltart and D.G. Shand, Br. Med. J., 3 (1970) 731-734.
12 L.D. DeLeve, L. Endrenyi and F.H.H. Leenen, J. Clin. Pharmacol., 25
    (1985) 182-186.
13 D.G. McDevitt and D.G. Shand, Clin. Pharmacol. Ther., 18 (1975) 708-713.
14 J.F. Mullane, J. Kaufman, D. Dvornik and J. Coelho, Clin. Pharmacol.
    Ther., 32 (1982) 692-700.
15 F.E. Okupa, T.K. Daneshmend, E. Shrosbree, C.J.C. Roberts, Clin.
    Pharmacol. Ther., 29 (1981) 434-439.
16 R.G. Shanks, S.G. Carruthers, J.G. Kelly and D.G. McDevitt, Postgraduate
    Med. J., 53 (Suppl. 3) (1977) 70-73.
17 A. Wellstein, D. Palm, J.H. Matthews and G.G. Belz, Meth. and Find.
    Exptl. Clin. Pharmacol., 7 (1985) 645-651.
18 A. Wellstein, D. Palm, G.G. Belz and H.F. Pitschner, Drug Res., 35 (1985)
    2-6.
19 O.E. Brodde, ISI Atlas of Science: Pharmacology, 1 (1987) 107-112.
20 E.M. Brown, S.A. Fedak, C.J. Woodard, G.D. Aurbach and D. Rodbard, J.
    Biol. Chem., 251 (1976) 1239-1246.
21 P.B. Molinoff, Drugs, 28 (Suppl 2) (1984) 1-15.
22 H.J. Motulsky and P.A. Insel, N. Engl. J. Med., 307 (1982) 18-29.
23 L.T. Williams and R.J. Lefkowitz (Editors), Receptor Binding in
    Adrenergic Pharmacology, Raven Press, New York, 1978.
24 O. Arunlakshana and H.O. Schild, Br. J. Pharmacol., 14 (1959) 48-58.
25 H.O. Schild, Brit. J. Pharmacol., 2 (1947) 251-258.
26 J.H. Gaddum, Pharmacol. Rev, 9 (1957) 211-218.
27 E.J. Ariens, Eur. J. Clin. Pharmacol., 26 (1984) 663-668.
28 P. Crevat-Pisano, C. Hariton, P.H. Rolland and J.P. Cano, J. Pharmaceut.
    Biomed. Anal.4 4 (1986) 697-715.
29 J.M. Ferkany Life, Sci., 41 (1987) 881-884.

278

30 M. Perret and P. Simon, J. Pharmacol. (Paris), 15 (1984) 265-286.
31 A. Wellstein, D. Palm, G. Wiemer, M. Schäfer-Korting and E. Mutschler, Eur. J. Clin. Pharmacol., 27 (1984) 545-553.
32 M. Staehelin, P. Simons, K. Jaeggi and N. Wigger, J. Biol. Chem., 258 (1983) 3496-3502.
33 A. Wellstein, D. Palm, G.G. Belz and H.F. Pitschner, Eur. J. Clin. Pharmacol., 29 (1985) 131-147.

34 A. Wellstein, D. Palm and G.G. Belz, J. Cardiovasc. Pharmacol., 8 (Suppl. 11) (1986) S36-S40.
35 G. Wiemer, A. Wellstein, D. Palm, H.M. v. Hattingberg and D. Brockmeier, Naunyn-Schmiedeberg´s Arch. Pharmacol., 321 (1982) 11-19.
36 A. Wellstein, B. Jablonka, G. Wiemer and D. Palm, Pol. J. Pharmacol. Pharm., 36 (1984):261-269.
37 X.L. Wang, M. Brinkmann and O.E. Brodde, Eur. J. Pharmacol., 114 (1985) 157-165.
38 D.G. McDevitt, M. Frisk-Holmberg, J.W. Hollifield and D.G. Shand, Clin. Pharmacol. Ther., 20 (1976) 152-157.
39 G. Sager, O.G. Nilsen and S. Jacobsen, Biochem. Pharmacol., 28 (1979) 905-911.
40 D. Hellenbrecht and J. Enenkel, Drug Res., 34 (1984) 980-983.
41 W.A. Ritschel, Drug Intell. Clin. Pharm., 14 (1980) 746-756.
42 A. Wellstein and D. Palm, Eur. J. Clin. Pharmacol., 29 (1985) 293-300.
43 K. Sriwatanakul and S.R. Nahorski, Eur. J. Pharmacol., 66 (1980) 169-178.
44 A. Wellstein, D. Palm, G.G. Belz, G. Leopold, K.U. Bühring and J. Pabst, J. Cardiovasc. Pharmacol., 8 (Suppl. 11) (1986) S41-S45.
45 G. Johnsson and C.G. Regardh, Clin. Pharmacokin., 1 (1976) 233-263.
46 A. Wellstein, D. Palm, Meth. and Find. Exptl. Clin. Pharmacol., 6 (1984) 641-644.
47 H. Spahn, A. Wellstein, G. Pflugmann, E. Mutschler and D. Palm (1987) submitted for publication.
48 C.F. George, T. Fenyvesi, M.E. Conolly and C.T. Dollery, Eur. J. Clin. Pharmacol., 4 (1972) 74-76.
49 G.P. Jackman, A.J. McLean, G.L. Jennings and A. Bobik, Clin. Pharmacol. Ther., 30 (1981) 291-296.
50 L.S. Olanoff, T. Walle, U.K. Walle, T.D. Cowart, T.E. Gaffney, Clin. Pharmacol. Ther., 35 (1984) 755-761.
51 B. Lemmer and K. Bathe, J. Cardiovasc. Pharmacol., 4 (1982) 635-644.
52 A. Wellstein, H. Küppers, H.F. Pitschner and D. Palm, Eur. J. Clin. Pharmacol., 31 (1987) 419-422.
53 D. Brockmeier, A. Wellstein, M.T. Verho, D. Palm, X. Int. Congress of Pharmacology, IUPHAR, Sydney (1987) Abstract
54 J.M. Cruickshank and G. Neil-Dwyer, Eur. J. Clin. Pharmacol. 28 (Suppl) (1985) 21-23.
55 Dost, Der Blutspiegel, Springer, 1956.

Chapter 7

DETERMINATION OF OPTICAL ISOMERS OF BETA-BLOCKERS

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7.1   INTRODUCTION

The beta receptor blocking drugs were designed to antagonize the binding and therefore the physiological actions of the neurotransmitter norepinephrine at the beta adrenergic receptor.   Since beta receptor binding for norepinephrine is highly stereoselective for the (-)-enantiomer[1], it is perhaps not surprising that beta receptor blocking drugs show analogous stereoselectivity. This has been shown to be the case for all beta blocking drugs, although the stereoselectivity for a given antagonist, depending upon its structure, can vary widely with a (-)/(+) enantiomer ratio for the binding affinity to the beta receptor being about 10 for atenolol and greater than 1000 for pindolol (ref. 1).

Considering the enantiomeric selectivity associated with beta receptor binding and the therapeutic actions of the beta receptor antagonists, it is of practical concern that almost all of these drugs are used clinically as their racemates.   It is possible that stereoselective processes, e.g. absorption, metabolism, distribution and excretion, can dramatically alter the stereochemical composition of the beta blocking drugs at their sites of action. Pharmacokinetics and therapeutic drug level monitoring have been extensively used to optimize therapy with these drugs.   However, until recently such measurements of drugs in plasma and other fluids and tissues have not been able to distinguish between the (-)-, or active, and the (+)-enantiomer. Thus, the true levels of active drug have been unknown.   Separation of individual enantiomers in biological samples after administration of racemic drugs is, however, no longer an insurmountable problem.   Extensive research in analytical chemistry, in particular over the last several years, has provided many approaches.

In this chapter we will discuss the various techniques that have been used

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[1]   As the (-)- and (+)-enantiomers of beta agonists and antagonists always refer to the active and inactive forms, respectively, whereas the R,S-nomenclature contains no such information, the (-),(+) nomenclature will be used in most cases.

to separate and measure the individual enantiomers of the beta blocking drugs and their metabolites. Three different approaches have been used, i.e. chromatographic methods, biological methods and methods based on stable isotope labeling. Many of these methods have found successful application to biological problems, whereas others have not yet been examined for their practical usefulness. The stereochemistry of the disposition of beta blocking drugs and their metabolites in man using the analytical techniques currently available has recently been reviewed (ref. 2).

7.2 CHROMATOGRAPHIC METHODS

With the chromatographic methods the most successful enantiomer separations have in general been achieved after chemical derivatization with a chiral (optically active) reagent to form diastereomer pairs, which are resolved using nonchiral stationary and mobile phases. This is exemplified by the reaction of (-)-N-trifluoroacetyl-1-prolyl chloride, (-)-TPC, with the secondary amino group of the beta blocking drugs, Fig. 7.1.
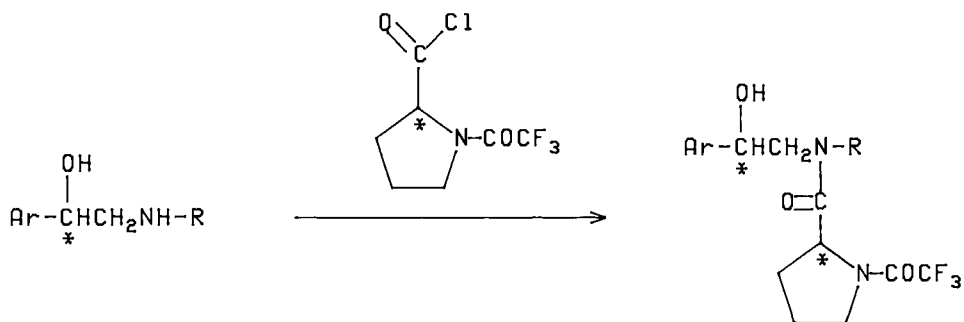
Fig. 7.1. Reaction of (-)-TPC with beta blocking drugs. Ar = aromatic ring substituent; R = aliphatic substituent; * = chiral carbons.

Before derivatization the enantiomers are mirror images of each other. Consequently, their physico-chemical properties in solution are identical and they are therefore not separable using nonchiral stationary and mobile phases. After chiral derivatization, which introduces a second asymmetric center in the molecule, the resulting diastereomer derivatives are no longer mirror images of each other. These derivatives have different physico-chemical properties in solution and may be separated on a nonchiral chromatographic system.

The beta blocking drug enantiomers may also be separated without prior

chiral derivatization using either chiral stationary or mobile phases. Differential interaction of the enantiomers with the asymmetric environment in the stationary or mobile phases in these chromatographic modes can achieve resolution of many of the beta blocking drug enantiomers.

The chromatographic system most successfully used is high performance liquid chromatography. Applications to thin-layer chromatography and gas chromatography will also be discussed.

7.2.1 High Performance Liquid Chromatography
7.2.1(a) HPLC – Diastereomers

(i) (-)-N-Trifluoroacetyl-1-prolyl chloride, (-)-TPC. The first separation of the enantiomers of a beta blocking drug by HPLC was for propranolol after chiral derivatization with (-)-TPC (refs. 3,4)(Fig. 7.1). The resolution of the diastereomers formed was complete on a reversed-phase nonchiral column, Fig. 7.2.
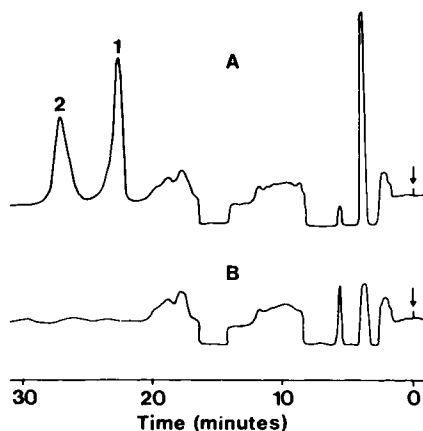
Fig. 7.2. Reversed-phase HPLC of plasma extracts of A. (-)-propranolol (1), 12.3 ng/ml, and (+)-propranolol (2), 8.9 ng/ml, and B. blank plasma after derivatization with (-)-TPC (ref. 3).

The use of fluorometric detection permitted quantitative determination of the enantiomers in blood plasma after therapeutic oral doses of racemic drug. The minimum detectable concentration of the propranolol enantiomers in plasma, after extraction with organic solvent, was claimed to be about 1 ng/ml. The derivatization was performed in chloroform or methylene chloride with triethylamine as a catalyst and was complete after 15 min at room temperature. The reaction mixture was washed with an organic/aqueous mixture and the

organic layer evaporated and reconstituted in mobile phase prior to HPLC. Hermansson and von Bahr (ref. 3) used commercially available (-)-TPC and noted some racemization of the reagent. Silber and Riegelman (ref. 4) prepared their own (-)-TPC. Very little racemization occurred. This latter method led to an investigation of the stereochemistry of propranolol disposition in several subjects (ref. 5). The question of racemization of the (-)-TPC reagent with time was not further investigated, although experiences in our own laboratories showed that this can be a problem.

Derivatization with (-)-TPC was also applied to the analysis of the enantiomers of acebutolol and its main metabolite diacetolol in a preliminary communication (ref. 6).

(ii) N-Tertiary-butoxycarbonyl-L-amino acid anhydrides. Because of an apparent problem of racemization of the proline reagent another type of amino acid derived chiral reagent was developed for the separation of the propranolol enantiomers (ref. 7). The symmetrical anhydrides of tertiary-butoxycarbonyl-L-alanine and tertiary-butoxycarbonyl-L-leucine (Fig. 7.3) were prepared from the corresponding amino acids.
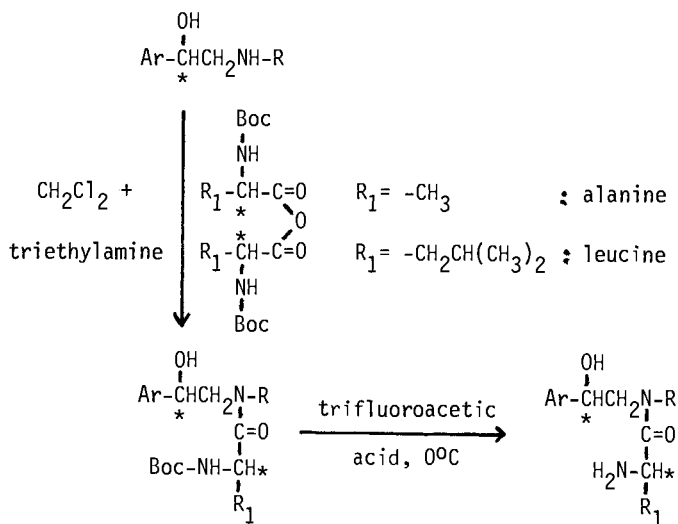
Fig. 7.3. Reaction of beta blocking drugs with symmetrical anhydrides of tertiary-butoxycarbonyl(Boc)-amino acids (ref. 7).

Propranolol, after extraction from plasma, was reacted with one of these anhydrides in methylene chloride with triethylamine as a catalyst at room temperature. Derivatization was complete in 5 to 30 min (longer time for the

leucine derivative).   Excess anhydride was hydrolyzed and the derivatives
formed were extracted with organic solvent and taken to dryness.   The residues
were treated with trifluoroacetic acid at 0°C to remove the tertiary-butoxy-
carbonyl group (Fig. 7.3).   After alkalinization the final derivatives were
extracted with ether and back-extracted into phosphoric acid prior to HPLC.
The propranolol diastereomer derivatives were separated by reverse-phase HPLC
using a mobile phase containing N,N-dimethyloctylamine in order to improve
peak symmetry.   The optimum separation factor achieved for the L-leucine
derivative was 1.70 compared to 1.30 for the L-alanine derivative and 1.20 for
the L-proline derivative discussed above (ref. 3).   No racemization occurred.
The sensitivity was the same as for the (-)-TPC derivatives and plasma enan-
tiomer levels of propranolol could be accurately measured after therapeutic
oral as well as intravenous doses of racemic drug (ref. 8).

The same method was applied to the determination of the enantiomers of
alprenolol and metoprolol (ref. 9) using the L-leucine derivatives.   Baseline
resolution was obtained on a 10 cm C_{18}-column without the addition of N,N-
dimethyloctylamine, Fig. 7.4.   No racemization was noticed.   A minor modifica-
tion of this method was used for studies of the stereoselective disposition of
metoprolol in man (ref. 10).
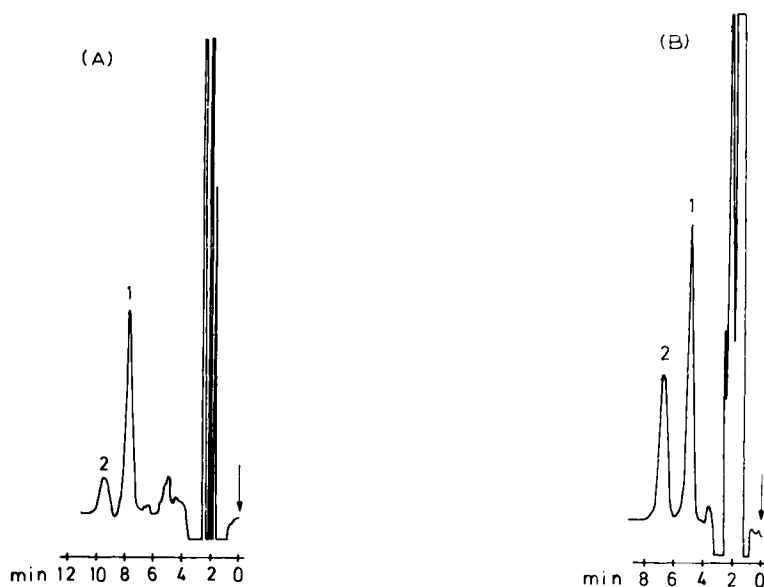
Fig. 7.4.   Reversed-phase HPLC of plasma extracts of A. (-)-alprenolol (1),
6.6 ng/ml, and (+)-alprenolol (2), 1.6 ng/ml, and B. (-)-metoprolol (1), 22.5
ng/ml, and (+)-metoprolol (2), 14.2 ng/ml, after derivatization with tertiary-
butoxycarbonyl-L-leucine (ref. 9).

(iii) <u>Phenylethyl isocyanate and phenylethyl isothiocyanate</u>. Phenylethyl isocyanate (PEI) was introduced as another alternative to (-)-TPC for chiral derivatization of beta blockers because of greater stability towards racemization. Both enantiomers of this reagent are commercially available. Several groups have shown that urea derivatives are formed (reaction with the secondary amino group exclusively), Fig. 7.5.
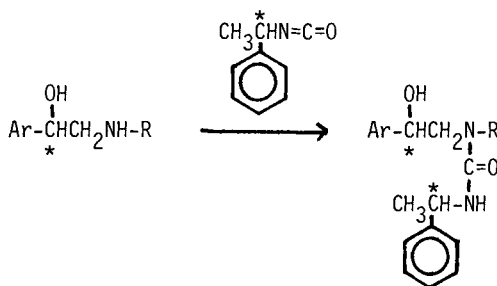
Fig. 7.5. Reaction of beta blocking drugs with (+)- or (-)- PEI.

The derivatization of propranolol with (+)-PEI (refs. 11,12) was considerably simpler than with tertiary-butoxycarbonyl-L-leucine above. After extraction of propranolol with methylene chloride the extract was dried with sodium sulfate and (+)-PEI was added at room temperature. After a short reaction time the solvent was removed by evaporation and the sample reconstituted in mobile phase prior to HPLC. Complete baseline separation of the diastereomer derivatives was achieved by reversed-phase chromatography in about 20 min.

When our laboratory tried to extend the (+)-PEI derivatization of propranolol to include the active metabolite 4-hydroxypropranolol (4-HOP) we noticed that in addition to reacting quantitatively with the amino group (+)-PEI also reacted with the phenolic group. This reaction was, however, incomplete. When the reaction mixture was shaken with 0.1 M hydrochloric acid, the phenolic derivative was hydrolyzed, producing the mono-(+)-PEI derivative only. The resolution of the 4-HOP diastereomers was, however, inadequate on a reversed-phase column. When switching to normal phase HPLC the resolution dramatically improved (R = 1.9). Also, a resolution of 1.5 was obtained for the propranolol diastereomers with a retention time of only 4 min. This method was used to assess the stereochemical composition of 4-hydroxypropranolol glucuronic acid and sulfate conjugates in human urine (ref. 13), Fig. 7.6.

When Dieterle and Faigle tried to determine the oxprenolol enantiomers
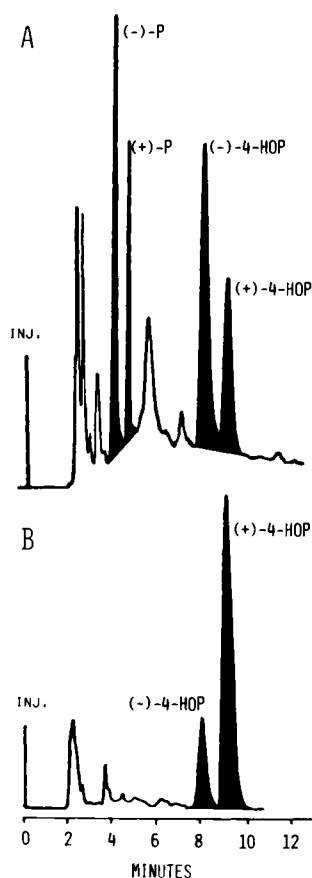
Fig. 7.6. Normal phase HPLC of (+)-PEI diastereomer derivatives of A. hydro-
lyzed glucuronic acid conjugates of propranolol (P) and 4-hydroxypropranolol
(4-HOP) and B. hydrolyzed sulfate conjugates of 4-HOP isolated from human urine
(ref. 13).

using (-)-TPC they did not get baseline separation. However, using (-)-PEI
and normal phase HPLC they succeeded (refs. 14,15). Because of inadequate
sensitivity by UV-detection they studied the kinetics of radioactive oxpren-
olol in dogs and man (100 µCi [14]C-oxprenolol). Prior to extraction of the
biological samples they added mg amounts of unlabeled racemic oxprenolol. The
eluate fractions corresponding to the two diastereomers (now visualized by
UV-detection) were collected and the radioactivity content in each peak
measured by liquid scintillation spectrometry.

Acebutolol and its metabolite diacetolol could also be separated into their
enantiomers after derivatization with (+)-PEI (ref. 16). The separation was
on a reversed-phase column using fluorescence detection. The minimum

detectable concentration was 50 ng/ml for spiked plasma samples. Baseline resolution was also obtained for pindolol, atenolol and acebutolol (ref. 17) on a reversed-phase column after derivatization with (-)-PEI. For pindolol interfering peaks were eliminated by extraction of plasma and urine samples at high pH, back-extraction into acid, alkalinization of the aqueous phase and another ether extraction. The limit of detection by fluorometry was 2 ng/ml plasma. Using this method stereoselective renal clearance of pindolol was shown (ref. 18).

Gal and Sedman (ref. 19) compared the resolution of the propranolol enantiomers on a reversed-phase column after derivatization with (+)-PEI and the corresponding sulfur analog (+)-1-phenylethyl isothiocyanate. The authors claimed that the thiourea derivatives were significantly better resolved than the urea derivatives. 2-Aminoethanol could be used to destroy excess reagent when the reagent peak interfered. No biological application was shown.

(iv) R-(-)-1-(1-Naphthyl)ethyl isocyanate. The rationale for using R-(-)-1-(1-naphthyl)ethyl isocyanate ((-)-NEI) as chiral derivatization reagent for beta blocking drugs is the higher UV-absorption and stronger fluorescence imparted by the naphthyl moiety compared to the phenyl moiety of the PEI reagent. This property of the NEI reagent was utilized to full advantage in pharmacokinetic studies of the betaxolol enantiomers (ref. 20). Blood concentrations of < 1 ng/ml of each enantiomer could be detected. The samples were extracted once at high pH after the addition of an internal standard. The evaporated residues were reacted with (-)-NEI in methanol for 1.5 hr at room temperature and excess reagent was evaporated, Fig. 7.7.
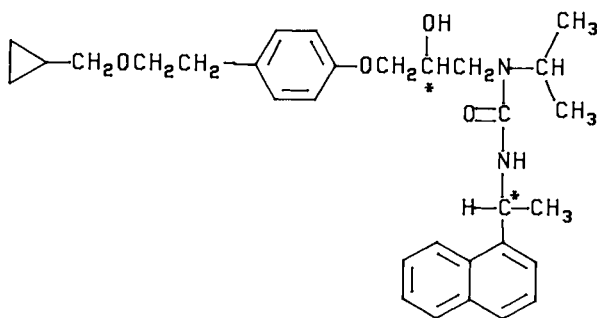
Fig. 7.7. Structure of the betaxolol (-)-NEI derivative (ref. 20).

Derivatization at 60°C did not increase the reaction rate, but instead gave rise to the formation of additional peaks, probably di-derivatives. The

derivatives were purified by chloroform extraction from pH 10 buffer. After evaporation of the chloroform the samples were dissolved in mobile phase and subjected to HPLC on a reversed-phase column. The addition of tetrahydrofuran and tetramethylethylenediamine improved peak symmetry and allowed baseline separation of the (-)-NEI betaxolol diastereomers in 12 min, Fig. 7.8. The separation was performed at 36°C in order to lower the head pressure caused by the 3 μm column packing. The blood concentrations of (+)- and (-)-betaxolol were measured in 3 subjects up to 36-72 hr after a single oral dose of 20 mg racemic betaxolol. The blood concentration-time curves were almost identical for the two enantiomers. Other racemic β-blocking drugs, such as propranolol, metoprolol and alprenolol, could also be resolved by this method by slightly modifying the tetrahydrofuran content in the mobile phase. All peaks eluted within 12 min.
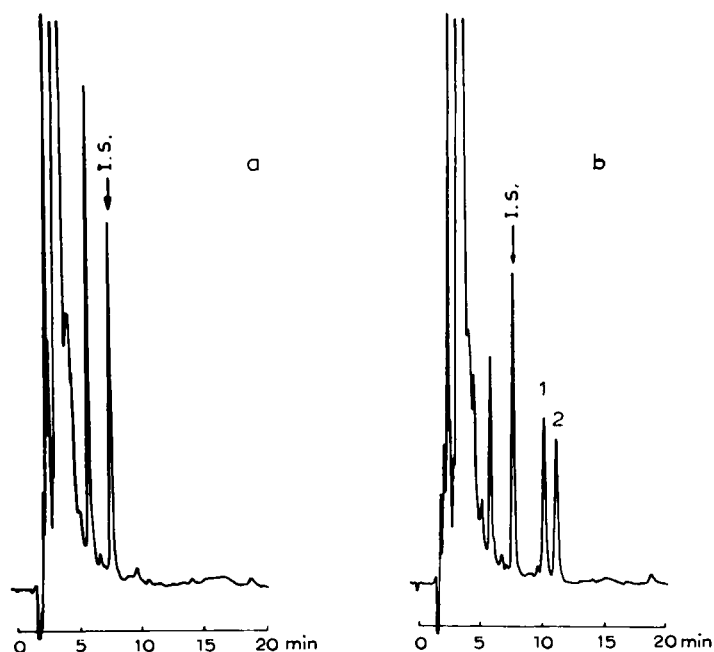
Fig. 7.8. Reversed-phase HPLC separation of blood extracts derivatized with (-)-NEI. (a) Blood spiked with 75 ng of the internal standard (I.S.), (-)-cicloprolol; (b) blood spiked with the internal standard as in (a) and with 20 ng/ml of each of the betaxolol enantiomers. Peaks: 1 = (-)-betaxolol; 2 = (+)-betaxolol (ref. 20).

(v) Tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate. 2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) (Fig. 7.9) was first
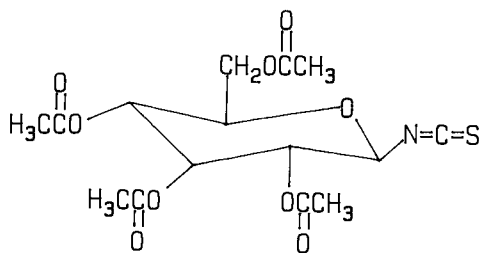
Fig. 7.9. Structure of 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothio-
cyanate (GITC).

introduced as a chiral derivatization reagent for the separation of amino acid
and catecholamine enantiomers (refs. 21,22) forming thiourea derivatives.
Sedman and Gal (ref. 23) investigated its usefulness for the separation of
beta blockers. The derivatization procedure for synthetic amounts of racemic
beta blockers was quite simple. The drugs (as free bases) were derivatized
with GITC in acetonitrile for 30 min at room temperature and the resulting
diastereomers were separated on a reversed-phase column. By varying the
acetonitrile content in the mobile phase a large number of beta blocking drugs
could be separated in 6-12 min with separation factors of 1.2 - 1.3, with the
(-)-enantiomers eluting first. No biological applications were shown.
Interestingly, when the 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate
was used, the elution order was reversed.

Our laboratory has recently used GITC derivatization for separation of the
enantiomers of several beta blockers and their metabolites in biological
fluids (refs. 24-28). These studies have confirmed the excellent properties
of the GITC derivatives. Using this methodology we have shown that the uptake
of atenolol by adrenergic storage granules and release by membrane depolariza-
tion are highly stereoselective (refs. 27,28). In these studies cells or
storage granules were incubated with racemic ^{3}H-atenolol. Prior to extraction
of the samples 25 μg of unlabeled racemic atenolol was added. After methylene
chloride extraction at pH 11 the extracts were taken to dryness and deriva-
tized for 5 min at room temperature with GITC in acetonitrile. Aliquots were
injected onto a reversed-phase column with UV-detection. The eluate fractions
corresponding to the GITC atenolol diastereomers were collected and the
radioactivity content measured by liquid scintillation spectrometry. A high
resolution (R = 2.9) was obtained with a short retention time (<6 min),
permitting rapid analysis.

(vi) (+)-1-(9-Fluorenyl)ethyl chloroformate. A new highly fluorescent

chiral reagent, (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC), was synthesized
for separation of amino acid enantiomers and optically active amines (ref.
29). Metoprolol was used as an example. Its reaction with FLEC is shown in
Fig. 7.10. Derivatization of samples was performed at neutral pH in
buffer/acetone. After 30 min hydroxyproline was added to consume excess
reagent. After 2 min the reaction mixture could be injected into the C_8
column. The hydroxyproline derivatives eluted before the metoprolol-FLEC
diastereomers. The separation was complete in 8 min (separation factor 1.1).
No biological applications were given.
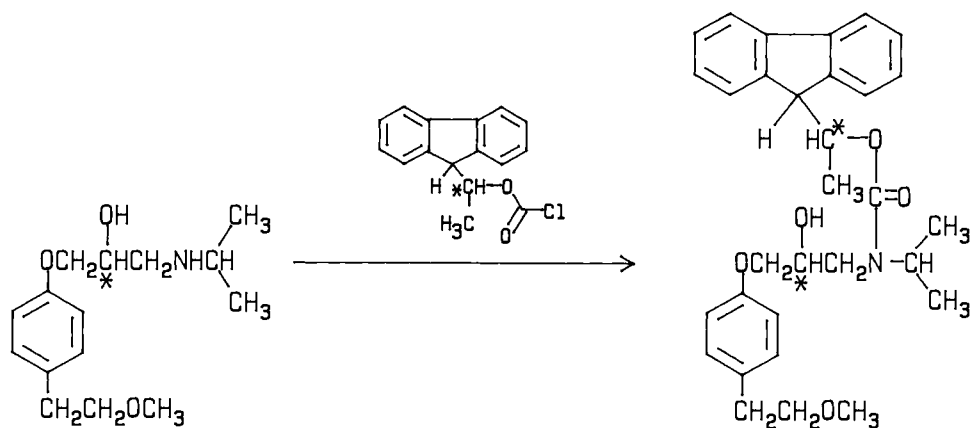
Fig. 7.10. Formation of FLEC derivative of metoprolol (ref. 29).

(vii) Substituted Tartaric Acid Anhydrides. The physicochemical proper-
ties of diastereomers become more similar with increasing distance between the
two chiral centers and the diastereomers are thus less likely to separate
chromatographically. As the side-chain hydroxyl group of beta blocking drugs
is directly attached to the chiral center, it would be advantageous to deriva-
tize the hydroxyl group. The problem is that any suitable chiral reagent
which reacts with the hydroxyl group also reacts with the amino group.
Lindner et al. (ref. 30) circumvented this problem in an elegant way by
blocking the amino group with a strong acid (e.g., trichloroacetic acid) to
form an ion-pair prior to derivatizing the alcohol function with substituted
tartaric acid anhydrides. To promote ion-pair formation the reaction had to
be carried out in an aprotic solvent. The general structure of the chiral
reagent is shown in Fig. 7.10a, where R can be acetyl, benzoyl, p-toluoyl,
methyl, ethyl or benzyl. An intramolecular ring structure for the 0,0-disub-
stituted tartaric acid monoesters of beta blockers is proposed and supported
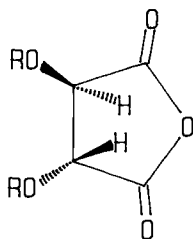
Fig. 7.10a. Structure of substituted tartaric acid anhydrides.

by NMR data. The proposed structure for the reaction product between (R,R)-O,O-diacetyl tartaric acid anhydride and propranolol is shown in Fig. 7.11.
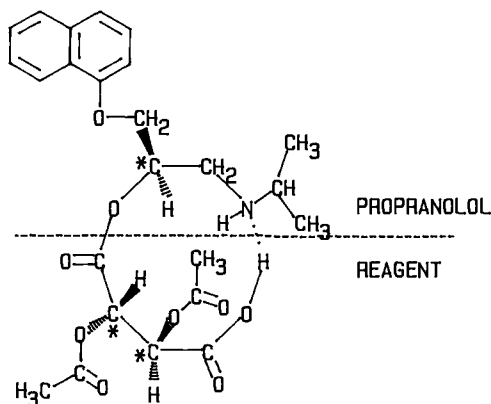
Fig. 7.11. Proposed structure of propranolol-(R,R)-O,O-diacetyl tartaric acid anhydride derivative (ref. 30).

On the analytical scale the derivatization was carried out as follows: The racemic drug (as free base) was dissolved in a dry, aprotic solvent (e.g. dichloroethane or acetone) and an excess of trichloroacetic acid and chiral reagent was added. The sealed tubes were kept at 50°C for several hours for complete reaction. After evaporation of the solvent, the samples were reconstituted in e.g. methanol and subjected to reversed-phase HPLC. The HPLC separation between the diastereomers was pH-dependent with a maximum around pH 6. The dibenzoyl reagent gave the best resolution with α-values of 2.6 - 5.0 for 15 beta blockers, i.e. considerably higher than for any of the chiral reagents reacting with the amino function.

When the enantiomers of propranolol were separated on a preparative scale an optical purity of 99.8% was obtained as verified by analytical HPLC. The chiral reagent was thus at least 99.8% pure and virtually no racemization occurred. The applicability of this method has yet to be tested for biological samples.

7.2.1(b)   HPLC - Chiral Mobile Phase

When enantiomeric amines are separated by HPLC with a mobile phase containing an optically active counter-ion such as (+)-10-camphorsulfonic acid, diastereomeric ion-pairs are formed (ref. 31). In order for these ion-pairs to be separated on a nonchiral column a number of conditions must be met. To promote ion-pair formation the mobile phase must have low polarity, e.g., dry methylene chloride with a low percentage of a lower alcohol. Dalgliesh's "three-point" rule (ref. 32) must also be met, i.e. there must be at least 3 points of interaction between the solute and the counter-ion. These basic prerequisites for separation seem to be met for beta blocking drugs by 1) electrostatic attraction, 2) hydrogen bonding between the hydroxyl group of the beta blocking side-chain and the oxo group of the camphorsulfonic acid (Fig. 7.12) and 3) hydrophobic interaction between the ring systems (ref. 31).
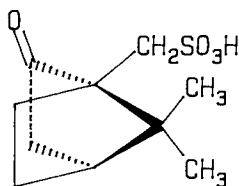
Fig. 7.12.   Structure of (+)-10-camphorsulfonic acid.

Separation factors of about 1.1 were obtained for alprenolol, metoprolol and propranolol using a mobile phase of 2 mM (+)-10-camphorsulfonic acid in methylene chloride/1-pentanol (199:1) on a LiChrosorb-DIOL column (31).

This technique has been applied to plasma concentration measurements of (+)- and (-)-metoprolol (refs. 33,34). The limit of detection was about 5 ng/ml at 227 nm excitation.

7.2.1(c)   HLPC - Chiral Stationary Phase

The separation of enantiomers on chiral stationary phases (CSPs), as with chiral mobile phases above, requires a minimum of three interactions between one of the enantiomers and the CSP (ref. 32). If these criteria are met, enantiomers may be separated directly on the CSP without chiral derivatization.

At least three types of CSPs have been investigated for the HPLC separation of
beta blockers: Pirkle, α_1-acid glycoprotein and cyclodextrin columns. All of
these columns are commercially available. Although their practical usefulness
for large numbers of biological samples remains largely unproven, they have
great potential.

(i)   Pirkle type CSP. Pirkle has introduced several types of covalently
and ionically bonded CSPs. However, only one CSP has been shown to resolve
the enantiomers of beta blocking drugs. It consists of (R)-N-(3,5-dinitroben-
zoyl)-phenylglycine ionically bonded to ɣ-aminopropyl silanized silica (ref.
35). The structure of this CSP is shown in Fig. 7.13. Interestingly, the
corresponding covalently bonded CSP gives no separation of these compounds.
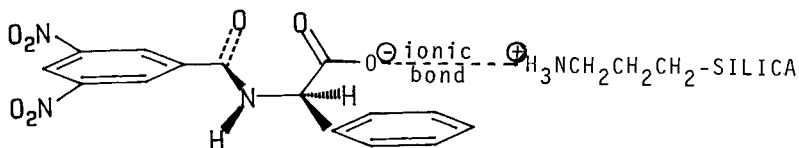
Fig. 7.13.   Structure of Pirkle ionic (R)-N-(3,5-dinitrobenzoyl)-phenylglycine
CSP.

The ionically bonded phenylglycine column was used by Pirkle for separation
of the enantiomers of propranolol, alprenolol and oxprenolol in mg quantities
(ref. 35). The mobile phase was hexane – 5% isopropanol. It was necessary to
acylate the beta blockers with lauroyl chloride to reduce the basicity of the
nitrogen and to shorten the elution times. The resolution was still incom-
plete with separation factors of 1.07-1.09. This type of separation system is
perhaps more useful for determination of enantiomeric purity and for prepara-
tive separation of enantiomers than for biological samples. Great care has to
be exercised in the use of this type of column, as the ionically bonded CSP is
easily washed off with polar mobile phases. Aqueous mobile phases can
obviously not be used.

Ionically bonded phenylglycine CSP was, however, shown to be useful for
blood level determinations of the propranolol enantiomers after chemical
derivatization with phosgene to form the cyclic oxazolidone derivatives (ref.
36) (Fig. 7.14). The derivatization procedure was quite simple. The ether
extract was reacted with phosgene for 30 sec at 0°C, evaporated and reconsti-
tuted. The sensitivity limit was about 0.5 ng/ml using excitation/emission
wavelengths of 290/335 nm. Using a mobile phase of hexane-isopropanol-aceton-
itrile (97:3:1) a separation factor of 1.09 was obtained, i.e. very long
elution times (about 50 min) were required for baseline resolution.
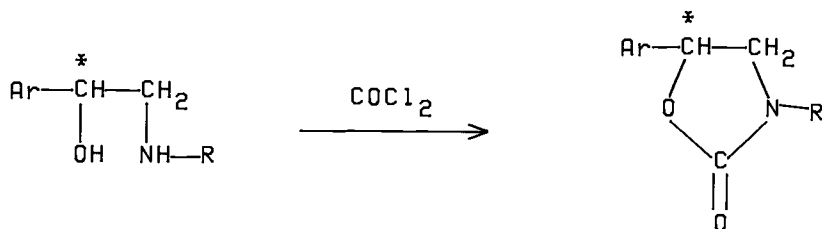
Fig. 7.14. Formation of phosgene derivatives of beta blocking drugs.

(ii) α_1-Acid Glycoprotein CSP. α_1-Acid glycoprotein (AGP) is an acidic protein (m.w. about 41,000), which is the main protein involved in the plasma protein binding of basic drugs. The stereoselectivity in plasma binding of propranolol was shown to be due to its stereoselective binding to AGP (ref. 37). Although it is not known how AGP interacts with cationic drugs, its sialic acid residues are believed to be involved. The AGP-CSP is prepared by ionic binding of a monolayer of protein to diethylaminoethyl silica. The protein is then oxidized, cross-linked through Schiff base formation and the enamines reduced to secondary amines (ref. 38). This CSP was developed by Hermansson (refs. 39,40).

Schill et al. (ref. 38) investigated the influence of different modifiers on the enantiomeric separation of a large number of cationic drugs, including some beta blockers. The metoprolol enantiomers were separated with 100% aqueous mobile phase ($\alpha = 1.64$); oxprenolol required the addition of ethanol ($\alpha = 1.25$); and propranolol ($\alpha = 1.13$) and pronethanol ($\alpha = 1.26$) varying amounts of N,N-dimethyloctylamine and 2-propanol.

Hermansson used a different approach to optimize the resolution of beta blocking drug enantiomers on AGP-CSP (ref. 41). The drugs were derivatized with phosgene prior to HPLC separation (reaction scheme, see Fig. 7.14). Derivatization dramatically improved the resolution of the enantiomers. Baseline or near-baseline resolution was obtained for alprenolol, oxprenolol and pindolol (α about 1.6) using a 3 cm column with phosphate buffer - 10% 2-propanol as the mobile phase. Propranolol had the remarkable separation factor of 5.7. The metoprolol enantiomers were not resolved with this mobile phase. By excluding the 2-propanol a separation factor of 1.95 was, however, obtained.

To our knowledge the AGP-CSP has not been used for beta blocking drugs in biological samples. One advantage of this CSP compared to the Pirkle CSP is better stability; aqueous mobile phases can be used. One drawback is that the column efficiency appears quite low and that the column is easily overloaded.

(iii) Cyclodextrin CSPs. A third type of CSP with potential usefulness for the separation of the enantiomers of beta blocking drugs is the cyclodextrins. The cyclodextrin-bonded phases are the first commercial CSPs to be used in a true reversed-phase mode (ref. 42). Cyclodextrins are doughnut-shaped molecules containing 6-12 glucose units bonded through α-(1,4) linkages, which are immobilized to support material. β-Cyclodextrin contains 7 glucose units, the optimum size for formation of inclusion complexes with (+)- and (-)-propranolol, see Fig. 7.15. Complexes of different stability and thus retention on the column are formed. In the (+)-propranolol complex the nitrogen atom is ideally placed for hydrogen bonding to hydroxyl groups in the cyclodextrin, whereas the nitrogen atom of (-)-propranolol is less favorably placed and thus less retained on the CSP. The propranolol and metoprolol enantiomers were at least partially resolved using two 25 cm β-cyclodextrin columns in series (ref. 42).
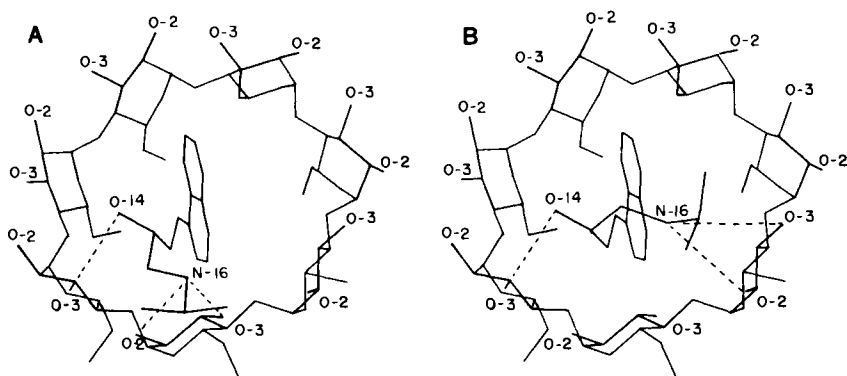
Fig. 7.15. Computer projections of inclusion complexes of (A) (+)-propranolol and (B) (-)-propranolol in β-cyclodextrin, from x-ray crystallographic data. Dotted lines represent potential hydrogen bonds. The configurations shown represent the optimal orientation of each isomer on the basis of the highest degree of hydrogen bonding and complexation. O-14 is the β-hydroxyl oxygen atom and N-16 the side-chain nitrogen atom (ref. 42).

7.2.2 Gas Chromatography

Two general approaches for separation of beta blocking drug enantiomers by gas chromatography (GC) are available: 1) formation and separation of diastereomers and 2) direct separation of enantiomers using chiral stationary phases.

Several of the chiral reagents used in the formation of diastereomers for HPLC separation are also amenable to GC analysis, although remaining polar groups, such as the β-hydroxyl group, need to be derivatized with a nonchiral

reagent to yield more volatile and stable products. Thus, a number of beta blockers have been derivatized with either N-trifluoroacetyl-(-)-prolyl chloride, (-)-TPC (see Section 7.2.1(a)(i)), or N-heptafluorobutyryl-(-)-prolyl chloride, (-)-HPC, and subsequently trimethylsilylated with N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA)(refs. 43,44), Fig. 7.16.

$$OTMS$$
$$Ar-CHCH_2N-R$$
$$*$$
$$O$$
$$*\quad N-COC_3F_7$$
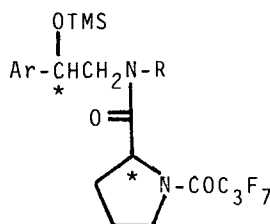
Fig. 7.16. Chiral derivatization of beta blocking drugs with (-)-HPC followed by trimethylsilylation (TMS) with BSTFA for enantiomer separation by GC.

The beta blockers as their free bases were derivatized with (-)-TPC or (-)-HPC in ethyl acetate and washed with dilute sodium hydroxide. The organic phase was evaporated to dryness and the β-hydroxyl group of the diastereomers derivatized with BSTFA in benzene. The reaction mixture was analyzed directly by GC. The silicone stationary phase OV-225 was used for both support-coated open-tubular and packed columns. As expected the capillary columns gave considerably better resolution of the diastereomers.

(-)-HPC gave better resolution than (-)-TPC of the enantiomers of alprenolol, oxprenolol, atenolol, pindolol, propranolol and its metabolite 4-hydroxypropranolol. The identity of the derivatives was confirmed by GC/MS. In a biological application the determination of relatively low concentrations (about 50 ng/ml) of propranolol used electron capture detection (ref. 44). The question of partial racemization (see Section 7.2.1(a)(i)) was not addressed.

In a short communication it was reported that the enantiomers of propranolol could also be resolved by GC after chiral derivatization with another useful reagent, (+)-1-phenylethyl isocyanate, (+)-PEI (see Section 7.2.1(a)(iii)). The resolution was 1.49 on a packed GC column and as high as 4.15 on a fused capillary column (ref. 12). Interestingly, the resolution was completely abolished when the β-hydroxyl group was trimethylsilylated.

As far as we can tell, only one chiral stationary phase, polysiloxane XE-60-L-valine-(R)-α-phenylethylamide, has been used for enantiomer separation of beta blocking drugs by GC. The glass capillary column was first coated with Silanox, then with XE-60-L-valine-(R)-α-phenylethylamide (ref. 45).

Several β-blockers could be separated after treatment with heptafluorobutyric (HFB) anhydride to form the di-HFB derivatives, although the α-values obtained were modest, Fig. 7.17.   Interestingly, beta blockers with an N-tertiary butyl instead of an N-isopropyl substituent could not be resolved.
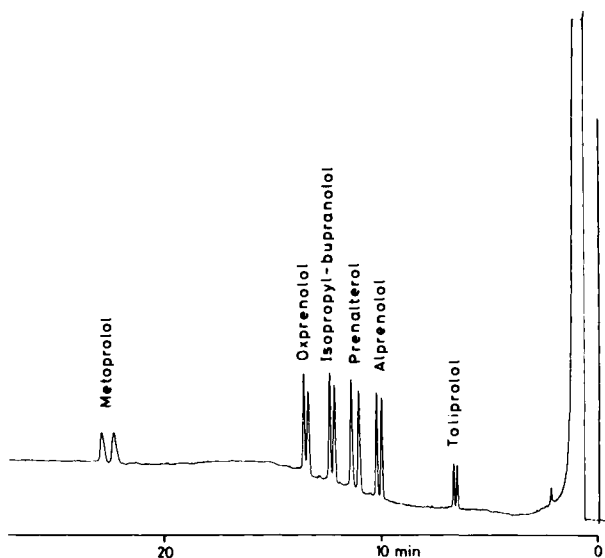
Fig. 7.17. GC separation of the enantiomers of beta blocking drugs on a chiral capillary   column   (polysiloxane   XE-60-L-valine-(R)-α-phenylethylamide)   as N,O-heptafluorobutyryl derivatives. Column temperature: 150°C for 5 min, then temperature programming at 1.5°C/min to 160°C (ref. 45).

Similar enantiomeric separation by GC using this chiral column was obtained when the beta blockers were derivatized with phosgene (reaction see Fig. 7.14) (ref. 46).   The oxazolidinone derivatives were more stable than the HFB derivatives. In contrast to the HFB derivatives the oxazolidinone derivatives of beta blockers with an N-tertiary butyl substituent could also be resolved into its diastereomers.

A slight modification of the phosgene derivatization method was used for the separation of the enantiomers of metoprolol and two of its metabolites on the same type of chiral capillary column (ref. 47). The oxazolidinone derivative of α-hydroxymetoprolol was further derivatized with N,O-bis(trimethyl-silyl)acetamide to form the TMS derivative of the free hydroxyl group prior to GC analysis. The carboxylic acid function of the metoprolol acid oxazolidi-none derivative was treated with diazomethane to form the methyl ester prior to GC separation. The enantiomers of both metabolites could be resolved to

the same extent as the parent compound (α = 1.03). The retention times were rather long, 44 min, 65 min and 78 min for complete resolution of metoprolol, α-hydroxymetoprolol and metoprolol acid, respectively.

7.2.3 Thin Layer Chromatography

Because of limited sensitivity the usefulness of thin layer chromatography (TLC) for the resolution of optical isomers in biological fluids is limited. However, two applications of this separation technique have been shown: 1) as an aid in the development of other separation methods and 2) for determination of beta blocking enantiomers in urine.

Following chiral derivatization with (-)-1-(1-naphthyl)ethyl isocyanate, (-)-NEI (see Section 7.2.1(a)(iv)), it was shown that TLC could separate the enantiomers of a number of beta blocking drugs (ref. 48). To avoid interference with excess reagent this was destroyed prior to the separation by the addition of diethylamine. Complete resolution was obtained using high performance silica gel TLC plates with benzene/ether/acetone as the mobile phase. The mobility of the (+)-enantiomers was, as with HPLC, greater than that of the (-)-enantiomers.

TLC separation has been shown to be useful for the quantitative determination of the enantiomers of metoprolol, oxprenolol and propranolol in urine (ref. 49). Toluene extracts of urine samples were evaporated to dryness and derivatized with (+)-benoxaprofen chloride, Fig. 7.18, in methylene chloride in the presence of dry sodium carbonate.
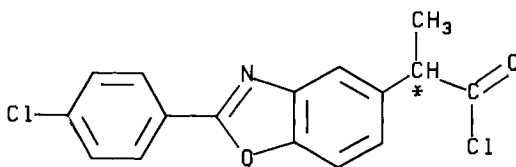
Fig. 7.18. Structural formula of benoxaprofen chloride used in the chiral derivatization of beta blocking drugs for TLC separation (ref. 49).

The samples were derivatized at room temperature overnight and the reaction stopped by the addition of methanol. No mention of the structures of the derivatives was made. After evaporation of the solvent the residue was dissolved in cyclohexane and aliquots applied to TLC plates (Kiesel gel 60). The plates were developed with toluene/acetone/ammonia and scanned at an excitation wavelength of 313 nm and emission wavelengths of 359-364 nm, Fig. 7.19. The mobility of the (-)-enantiomers was greater than that of the
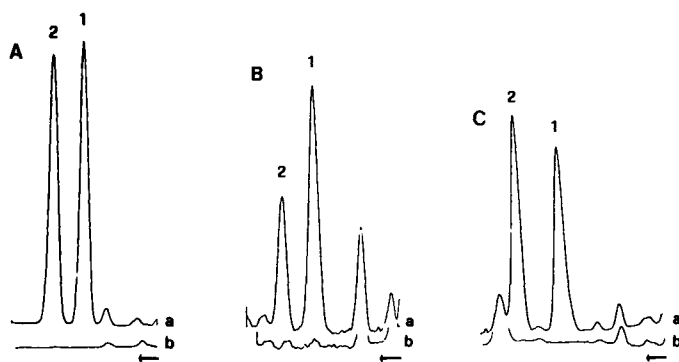
Fig. 7.19. TLC of metoprolol (A. 9.54 µg/ml), oxprenolol (B. 1.81 µg/ml) and propranolol (C. 1.67 µg/ml) after extraction from human urine and derivatization with (+)-benoxaprofen chloride using silica gel (Kiesel gel 60) plates. Mobile phase: toluene/acetone/ammonia (100:10:gaseous). Peaks: 1 = (+)-enantiomer; 2 = (-)-enantiomer. Traces: a = 0-2 hr urine; b = blank urine (ref. 49).

(+)-enantiomers. The detection limit was 100 ng/ml urine. The obvious advantage with TLC as compared to other separation methods is that a large number of samples can be run simultaneously using inexpensive equipment.

7.3 BIOLOGICAL METHODS

7.3.1 Radioimmunoassays

The objective of the radioimmunoassays (RIAs) is to measure circulating levels of parent drug. Advantages of these biological assays include high sensitivity, technical simplicity and speed. The first attempt to determine one enantiomer of a beta blocking drug in the presence of the other was done with an RIA in 1976 (ref. 50). Although many RIAs for beta blocking drugs have been developed since then, most of them have not focused on the active (-)-enantiomer. The use of RIAs based on polyclonal antibodies (i.e. antisera) for measurements of beta blocking drugs, including stereoselective assays, is described in Chapter 6.1.

More recently stereoselective monoclonal antibodies (MAbs) to propranolol were developed (ref. 51). As immunogen these authors coupled BSA with propranolol through the isopropyl group. The propranolol-BSA conjugate was used for immunization of BALB/c mice. Splenocytes from these mice were fused with myeloma cells and cultures secreting antibodies to propranolol isolated, cloned and finally propagated in vivo as ascites tumor in BALB/c mice. Ascites fluid as well as culture supernatant were used as antibody sources. RIAs were developed for two of the MAbs. One clone showed about 12 times higher affinity for (-)-propranolol compared to (+)-propranolol (Fig. 7.20),
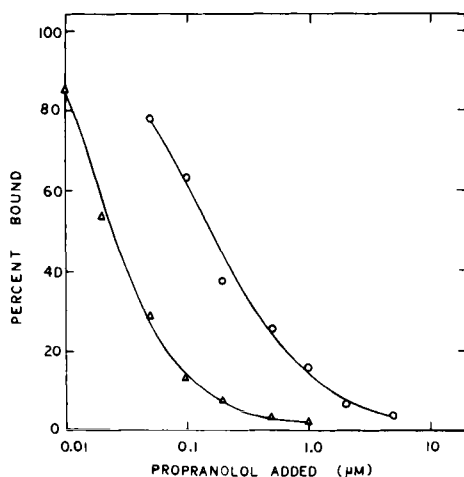
Fig. 7.20. Reaction of MAb from clone P-49 with the propranolol enantiomers.
(Δ), (-)-Propranolol; (o), (+)-propranolol (ref. 51).

even though racemic hapten was used. This MAb cross-reacted minimally with
alprenolol (4%) and not at all with other beta blockers tested. Cross-
reactivity of metabolites was not investigated.

Monoclonal anti-alprenolol antibodies have also been developed by immuni-
zation of mice with (±)-alprenolol (ref. 52) or (-)-alprenolol (ref. 53)
coupled to keyhole limpet hemocyanin. All cloned antibodies cross-reacted
with propranolol. One of four anti-(±)-alprenolol antibodies had a 20-fold
higher affinity for (-)-compared to (+)-propranolol (ref. 52). Three of four
anti-(-)-alprenolol antibodies had greater than 100-fold higher affinity for
(-)- compared to (+)-propranolol (ref. 53).

Although these monoclonal antibodies have been developed for characteriza-
tion of beta receptors and have not been tested for enantiomer level deter-
minations in biological samples, they could potentially be used for this type
of application. The development of MAbs with desired specificity requires a
major effort both with respect to synthesis of antigen and above all for
testing of a large number of clones. Once a suitable MAb has been found,
however, unlimited amounts of antibody with identical properties can be
produced. In contrast, the properties of polyclonal antibodies, i.e. anti-
sera, vary between batches.

A potential common problem with the RIAs is questionable specificity,
including interference from metabolites. This is particularly important when
the metabolism of the drug is not completely understood and should be tested
with an independent stereospecific assay.

7.3.2   Radioreceptor Assays

Whereas RIAs are based on the competition between unlabeled parent drug in a sample and radiolabeled ligand for binding to antibodies, radioreceptor assays (RRAs) are based on the competition for binding to a receptor preparation. As the β-adrenergic receptors by nature are very selective for the (-)-enantiomers of β-adrenergic receptor-blocking drugs, all RRAs for these drugs are quite selective for biologically active drug, i.e. the (-)-enantiomers, but also for any active metabolites that may be present, e.g., the (-)-enantiomer of 4-hydroxypropranolol. The use of RRAs for measurements of beta blocking drugs is described in Chapter 6.2.

7.4   STABLE ISOTOPE METHODS

The use of artificial stable isotope-labeled pseudoracemates as dosage forms in combination with gas chromatography-mass spectrometry (GC/MS) is a completely different approach to quantitative analysis of enantiomers of drugs and their metabolites in biological systems. This research technique was used in a study of propranolol in the dog, demonstrating for the first time stereoselectivity in the oral clearance of a beta blocking drug (ref. 54). The use of this technique is shown in a more recent study in man (ref. 55).

In this study it was of interest to determine the kinetics of simultaneously administered intravenous and oral doses of propranolol. As the oral dose but not the intravenous dose kinetics show marked stereoselectivity, the enantiomers after the racemic oral dose needed to be separated from each other as well as from the dose given intravenously. The intravenous dose chosen was regular unlabeled (±)-propranolol (8 mg). The oral dose was an artificial 50:50 mixture of dideuterium-labeled (-)-propranolol and hexadeuterium-labeled (+)-propranolol (40 mg of each). Plasma samples were extracted at pH 12 with benzene after the addition of the internal standard (±)-oxprenolol. The three forms of propranolol and oxprenolol were derivatized with trifluoroacetic anhydride to form the N,O-trifluoroacetyl derivatives prior to analysis by GC/MS using selected ion monitoring. The structures of the derivatives and their molecular weights are shown in Fig. 7.21.

The three forms of propranolol had about the same retention time by GC but were completely separated by selected ion monitoring based on their different molecular weights ($\underline{m}/\underline{z}$ 451, 453 and 457), Fig. 7.22. The internal standard oxprenolol had the same molecular weight as d_6-(+)-propranolol (($\underline{m}/\underline{z}$ 457) but had a shorter retention time. At 10 min after the administration of the drugs the plasma as expected had its highest concentration of the intravenous dose (d_0-(±)-propranolol). Of the oral dose only d_2-(-)-propranolol had appeared in plasma at this time. At 150 min both forms of the oral dose were present with the concentration of d_2-(-)-propranolol ($\underline{m}/\underline{z}$ 453) exceeding that of
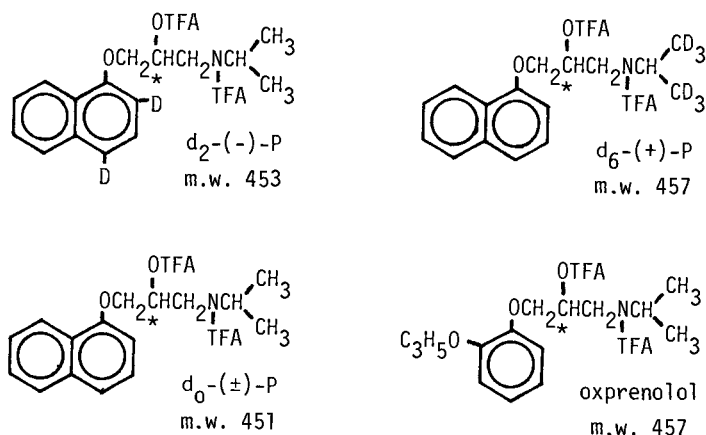
Fig. 7.21. Structures of the N,O-trifluoroacetyl (TFA) derivatives of the oral dose (50:50 mixture of d_2-(-)-propranolol and d_6-(+)-propranolol), the intravenous dose (d_0-(±)-propranolol) and the internal standard ((±)-oxprenolol) together with their molecular weights.

d_6-(+)-propranolol ($\underline{m}/\underline{z}$ 457).

As can be seen in Fig. 7.22 the retention time was only about 3 min, thus the speed of analysis greatly exceeds that of other chromatographic techniques for enantiomer separations. The sensitivity was about 1 ng/ml plasma, which could probably be further increased using chemical rather than electron impact ionization. The specificity associated with GC separation and subsequent mass specific detection is virtually absolute.

It is important to consider the site of the deuterium labeling so as to avoid a stable isotope effect that may occur during metabolism. However, neither the two deuterium atoms in d_2-(-)-propranolol nor the six deuterium atoms in d_6-(+)-propranolol described above are involved in the initial metabolic oxidations of propranolol. Although propranolol is extensively hydroxylated in the 4'-position, this involves a 3',4'-epoxidation (refs. 56,57), thus does not involve breakage of a carbon-deuterium bond. For side-chain oxidation of propranolol the initial oxidative attack occurs at the methine of the isopropyl group. The six deuterium atoms in side-chain labeling are thus not involved in oxidative metabolism. To test whether a stable isotope effect is involved or not, it is common to switch the labeling of the individual enantiomers. If identical kinetics are obtained, such an effect can be excluded (refs. 54, 58-60). Preparation of the labeled enantiomers described above is according to simple procedures, involving preparative resolution of the propranolol enantiomers and deuterium-hydrogen exchange (ring labeling) or synthesis of hexadeuterium-labeled propranolol and preparative resolution of the enantiomers (ref. 58). These approaches described for
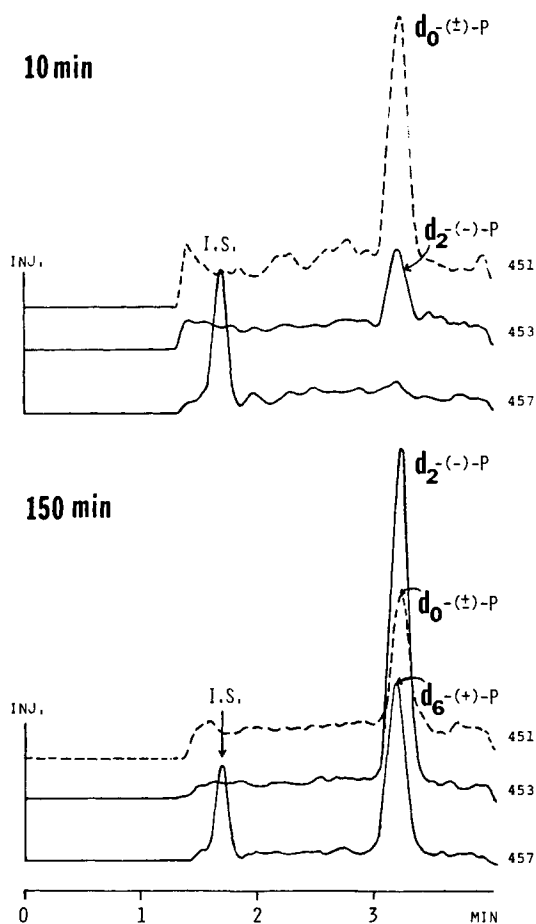
Fig. 7.22. Selected ion monitoring GC/MS of different forms of propranolol after extraction from human plasma and trifluoroacetylation. Tracings are shown at 10 and 150 min after dosing. Intravenous dose: d_0-(±)-propranolol, d_0-(±)-P ($\underline{m/z}$ 451). Oral dose: 50:50 mixture of d_2-(-)-propranolol, d_2-(-)-P ($\underline{m/z}$ 453) and d_6-(+)-propranolol, d_6-(+)-P ($\underline{m/z}$ 457). Internal standard (I.S.): (±)-oxprenolol ($\underline{m/z}$ 457)(Walle $\underline{et}$ $\underline{al}$., unpublished).

propranolol should be useful for other beta blockers.

The stable isotope-labeled pseudoracemate approach is not only useful for the parent drug but equally useful for the beta blocking drug metabolites. The stereochemical composition of all metabolites separable by GC should be possible to determine in a fashion identical to Fig. 7.22. This is shown in Fig. 7.23. The two top tracings show the stereochemical composition of propranolol glycol and N-desisopropylpropranolol in human urine after hydroly-sis with β-glucuronidase following oral administration of the pseudoracemate d_2-(+)-propranolol/d_0-(-)-propranolol (ref. 59). The sample was extracted and

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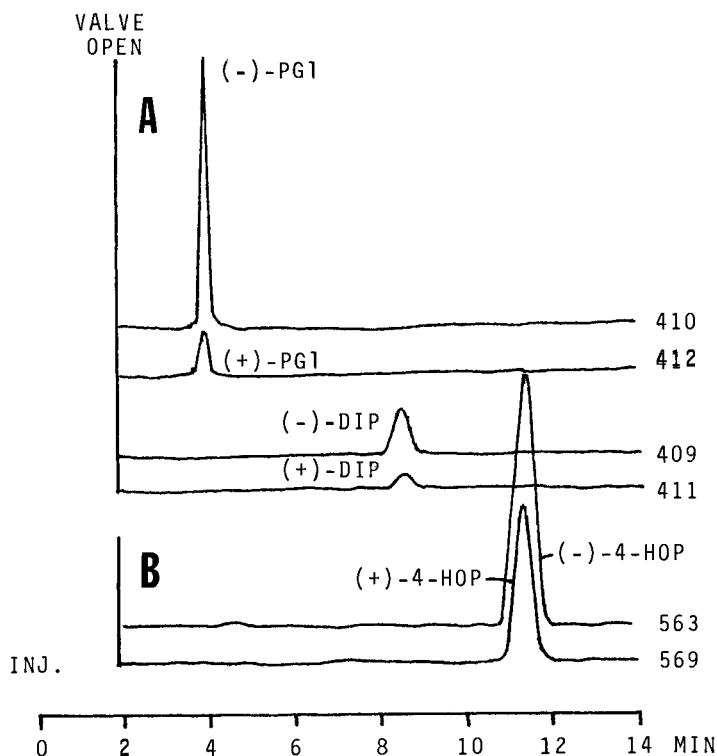
Fig. 7.23. Selected ion monitoring GC/MS of the enantiomers of A. propranolol glycol (PG1) and N-desisopropylpropranolol (DIP) after oral doses of d_2-(+)-propranolol/d_0-(-)-propranolol and of B. 4'-hydroxypropranolol (4-HOP) after oral doses of d_6-(+)-propranolol/d_0-(-)-propranolol in man. The metabolites were extracted from urine after beta-glucuronidase hydrolysis and then tri-fluoroacetylated (Walle et al., unpublished).

derivatized as propranolol above and the mass spectrometer was focused on the molecular ions (m/z 412/410 and 411/409, respectively). Note a very large enrichment of the (-)-enantiomers in both of these metabolites. In the bottom tracing is shown the stereochemical composition of 4'-hydroxypropranolol in human urine after hydrolysis of the glucuronide. In this case the oral dose was the pseudoracemate d_6-(+)-propranolol/d_0-(-)-propranolol (ref. 59). For this metabolite there is also a greater enrichment of the (-)-enantiomer. Thus, it is not necessary to develop separate conditions to resolve metabolite enantiomers as is usually the case with HPLC methodology. Similar stable isotope based approaches have been used also in other studies (refs. 61-65).

Nelson et al. introduced an improved stable isotope labeling approach in mechanistic studies of the stereochemistry of propranolol metabolism in

animals and man (refs. 66-69). Whereas in the examples given above d_6 (side-chain) labeling could only be used to monitor ring oxidation metabolites and d_2 (ring) labeling could only be used to monitor side-chain oxidation metabolites, Nelson prepared a pseudoracemate with two deuterium atoms in the 3-position of the n-propyl side-chain, Fig. 7.24. Since these deuterium atoms will not be lost during metabolism, both ring and side-chain oxidation products can be monitored by selected ion monitoring GC/MS after extraction and derivatization procedures similar to those described above.
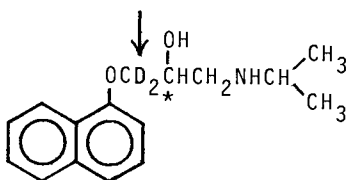
Fig. 7.24. Stable isotope labeling of propranolol enantiomers according to Nelson et al. (refs. 66-69).

The stereochemical composition of xibenolol and its three major metabolites (Fig. 7.25) was recently determined in both urine and plasma of man using similar methodology (ref. 60). In this study two stable isotope-labeled pseudoracemates were used in separate drug administrations, one containing
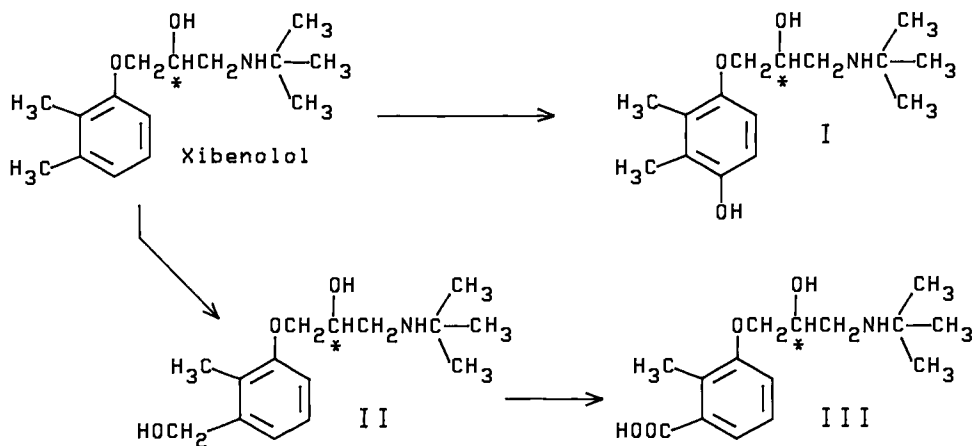
Fig. 7.25. Structure of xibenolol and its major metabolic products 1. 4'-hydroxyxibenolol, 2. 3'-hydroxymethylxibenolol and 3. 3'-carboxyxibenolol (ref. 60).

five deuterium atoms in the n-propyl side-chain, the other nine deuterium atoms in the tertiary butyl group. Parent drug and metabolites were isolated by extraction, ion-exchange chromatography and TLC after internal standards had been added. Quantitation and stereochemical composition were accomplished by mass chromatography using electron impact ionization. The sensitivity was high for both parent drug and metabolites permitting accurate analysis of low plasma concentrations. No stable isotope effect was noticed. Interestingly, this study demonstrated considerably higher concentrations of the (-)-enantiomers of two active metabolites, 4'-hydroxyxibenolol and 3'-hydroxymethylxibenolol, than of the parent drug.

7.5   POLAR BETA BLOCKING DRUG METABOLITES

Among the chromatographic and stable isotope methods discussed above are many examples of separation of enantiomers of metabolites. These are metabolites which in general have similar physico-chemical properties as their parent drugs and thus are amenable to simple solvent extraction for their isolation from biological fluids and standard chemical derivatization prior to HPLC or GC/MS. There are, however, also very prominent polar metabolites of this class of drugs, the formation of which has been suggested to be stereoselective.

The enantiomers of glucuronic acid conjugates such as of propranolol and 4'-hydroxypropranolol have been determined indirectly after enzymatic removal of the glucuronic acid moiety (refs. 5,54). The validity of such methods should be confirmed by direct methods. These metabolites by virtue of being derivatives of β-D-glucuronic acid are diastereomer pairs and should therefore be possible to resolve intact by chromatographic methods. This has been done using reversed-phase HPLC. In in vitro experiments it only required precipitating the microsomal protein in the samples by the addition of acetonitrile (refs. 70,71). HPLC separation of the intact diastereomer glucuronides excreted in urine following oral doses of racemic propranolol required a purification scheme involving isolation by DEAE-Sephadex anion-exchange chromatography and desalting on an XAD-2 column. This was also applied to the alprenolol glucuronides (ref. 72). The intact diastereomer glucuronides of beta blockers can also be separated by GC after trifluoroacetylation (ref. 73).

Separation of the enantiomers of the sulfate conjugate of 4'-hydroxypropranolol has also been accomplished indirectly after enzymatic removal of the sulfate moiety (refs. 13,59). A more satisfactory direct approach is also possible. The intact sulfate conjugate, but not the corresponding glucuronic acid conjugate, can be quantitatively extracted from both plasma and urine by an ion-pair method using tetrabutyl ammonium as the counter ion (ref. 74). It

can then be derivatized with the chiral reagent GITC (cf. Fig. 7.9) and separated by reversed-phase HPLC (ref. 25).

Separation of the enantiomers of lactic acid metabolites, which are common for most beta blockers, has also been accomplished by stable isotope methods (refs. 58,59). The HPLC methods discussed above have not been applicable to these 2-hydroxy carboxylic acids. A chiral derivatization approach used for the enantiomeric separation of a nonsteroidal antiinflammatory drug by GC (ref. 75) was recently applied in our laboratory to HPLC separation of the enantiomers of a major propranolol metabolite, α-naphthoxylactic acid (NLA). After extraction of NLA with ethyl acetate at pH 2 from biological fluids, NLA was reacted with carbonyldiimidazole to activate the carbonyl group before reaction with (-)-α-methylbenzylamine in the presence of acetic acid. After 30 min dilute hydrochloric acid was added to hydrolyze excess carbonyl-diimidazole. The diastereomers were extracted into hexane, evaporated, recon-stituted in mobile phase and separated by reversed-phase HPLC (Walle et al., unpublished).

7.6 SYNTHETIC DIASTEREOMERIC DRUGS

Nadolol and labetalol differ from other beta blocking drugs in that they have more than one asymmetric center, see chemical structures in Fig. 7.26.
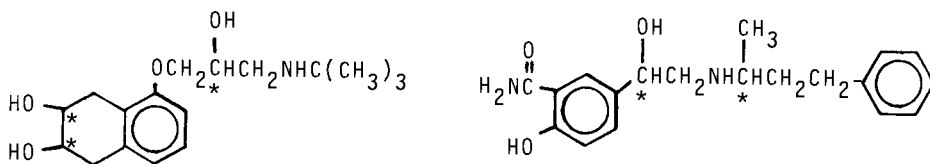
Fig. 7.26. Chemical structures of nadolol (left) and labetalol (right).

Nadolol has three asymmetric carbon atoms. However, as the two hydroxyl groups in the cyclohexene ring have a cis-configuration, nadolol exists as four stereoisomers as does labetalol with its two asymmetric carbon atoms. Thus, both of these drugs consist of two diastereomeric racemates, (+),(+)/(-),(-) and (+),(-)/(-),(+). The two racemates have different physicochemical properties and can be chromatographically separated into two peaks on a nonchiral column. Nadolol has thus been separated into its two racemates by reversed-phase (ref. 76) (Fig. 7.27) or cation-exchange HPLC (ref. 77). Racemate A consists of (+)-side-chain/(-)-ring and (-)-side-chain/(+)-ring nadolol, whereas racemate B consists of (+)-side-chain/(+)-ring and (-)-side-chain/(-)-ring nadolol (ref. 76). Racemate B is three times more potent as a beta blocker than racemate A (ref. 77).
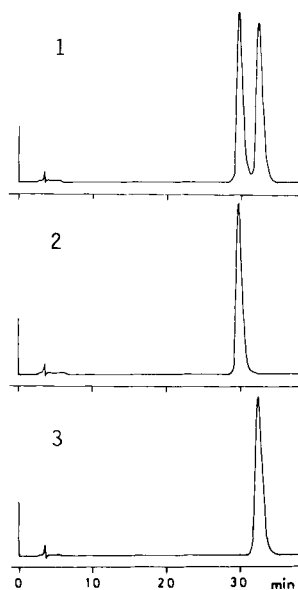
Fig. 7.27. Reversed-phase HPLC of nadolol diastereomers. 1 = Nadolol; 2 = racemate A; 3 = racemate B (ref. 76).

Labetolol has been separated into its racemic diastereomers by GC as the alkyl boronic acid di-derivatives (refs. 78,79), although not from biological samples. As the beta blocking activity resides in only one of the four stereoisomers (R,R) and two of the others have varying degrees of alpha blocking activities (S,R and S,S) (ref. 78), it would be highly desirable to measure the blood levels of all four isomers.

7.7 CONCLUSIONS

It appears clear from current literature that it is possible to determine the enantiomers of beta blocking drugs and most of their metabolites in biological fluids and tissues after administration of racemic drug by a number of analytical techniques. The two most important factors that must be considered when selecting a method are specificity and sensitivity. Judging from observations of techniques that have been applied to biological problems, both of these factors are fulfilled in a variety of methods utilizing chiral derivatization and HPLC with fluorometric detection. The more recently described chiral reagents are preferable. If the fluorescence for some drugs is not adequate, derivatization with a fluorescent reagent may be needed. With the proper choice of chiral reagent methods development is probably less time-consuming than for other techniques. Other modes of HPLC separation using

either chiral mobile or stationary phases have not yet been proven satisfactory for beta blocking drugs in general. More biological applications of these modes are needed in order to judge their practical usefulness. Although GC has proven capable of beta blocking drug enantiomer separations, it is surprising that this technique has not been used more often in biological applications. TLC in general lacks the sensitivity needed but may be quite useful in stereospecific methods development. Radioimmunoassays have the potential to be the simplest and most rapid technique for large numbers of samples, providing that their specificity can be established. The radio-receptor assay could be developed to a useful technique in therapeutic drug level monitoring, as it measures the binding of active enantiomer only (parent drug + metabolites) to the beta adrenergic receptors. The use of stable isotope labeled drugs and GC/MS, although requiring a substantial initial investment in instrumentation and chemical synthesis, as a research tool is superior to other techniques both with respect to specificity and sensitivity. As this technique requires administration of labeled drugs, it can obviously not be used in therapeutic conditions.

As it has been demonstrated that beta blocking drug enantiomers behave differently in the body both with respect to metabolism and binding to macro-molecules (2), it is important that therapeutic drug level monitoring is based on the pharmacologically active (-)-enantiomer. The analytical technology is clearly available. The analytical techniques described for current beta blockers should also be applicable to enantiomer separation of new drugs of this class.

ACKNOWLEDGEMENTS

REFERENCES

1 T.H. Morris and A.J. Kaumann, Naunyn-Schmiedeberg's Arch. Pharmacol., 327 (1984) 176-179.
2 T. Walle, J.G. Webb, E.E. Bagwell, U.K. Walle, H.B. Daniell and T.E. Gaffney, Biochem. Pharmacol., in press (1988).
3 J. Hermansson and C. von Bahr, J. Chromatogr., 221 (1980) 109-117.
4 B. Silber and S. Riegelman, J. Pharmacol. Exp. Ther., 215 (1980) 643-648.
5 B. Silber, N.H.G. Holford and S. Riegelman, J. Pharm. Sci., 71 (1982) 699-704.
6 M.G. Sankey, A. Gulaid and C.M. Kaye, J. Pharm. Pharmacol., 36 (1984) 276-277.

7 J. Hermansson, Acta. Pharm. Suec., 19 (1982) 11-24.
8 C. von Bahr, J. Hermansson and K. Tawara, Br. J. Clin. Pharmacol., 14 (1982) 79-82.
9 J. Hermansson and C. von Bahr, J. Chromatogr., 227 (1982) 113-127.
10 M.S. Lennard, G.T. Tucker, J.H. Silas, S. Freestone, L.E. Ramsay and H.F. Woods, Clin. Pharmacol. Ther., 34 (1983) 732-737.
11 M. Tsuru, J.A. Thompson, J.L. Holtzman, C.L. Lehrman, L. Mottanen and J.L. Holtzman, Clin. Pharmacol. Ther., 31 (1982) 275-276.
12 J.A. Thompson, J.L. Holtzman, M. Tsuru, C.L. Lerman and J.A. Holtzman, J. Chromatogr., 238 (1982) 470-475.
13 M.J. Wilson and T. Walle, J. Chromatogr., 310 (1984) 424-430.
14 W. Dieterle and J.W. Faigle, J. Chromatogr., 259 (1983) 311-318.
15 W. Dieterle, J.W. Faigle, W. Küng and W. Theobald, Xenobiotica 16 (1986) 181-191.
16 A.A. Gulaid, G.W. Houghton and A.R. Boobis, J. Chromatogr., 318 (1985) 393-397.
17 P.-H. Hsyu and K.M. Giacomini, J. Pharm. Sci., 75 (1986) 601-605.
18 P.-H. Hsyu and K.M. Giacomini, J. Clin. Invest., 76 (1985) 1720-1726.
19 J. Gal and A.J. Sedman, J. Chromatogr., 314 (1984) 275-281.
20 A. Darmon and J.P. Thenot, J. Chromatogr., 374 (1986) 321-328.
21 T. Kinoshita, Y. Kasahara and N. Nimura, J. Chromatogr., 210 (1981) 77-81.
22 N. Nimura, Y. Kasahara and T. Kinoshita, J. Chromatogr., 213 (1981) 327-330.
23 A.J. Sedman and J. Gal, J. Chromatogr., 278 (1983) 199-203.
24 S. Ward, R.A. Branch, T. Walle and U.K. Walle, Pharmacologist, 28 (1986) 137.
25 T. Walle, D.C. Christ, U.K. Walle and M.J. Wilson, J. Chromatogr., 341 (1985) 213-216.
26 D.D. Christ and T. Walle, Drug Metab. Dispos., 13 (1985) 380-381.
27 J.G. Webb, E.E. Bagwell, T. Walle and T.E. Gaffney, Circulation, 74 (1986) II-375.
28 E.E. Bagwell, J.G. Webb, T. Walle and T.E. Gaffney, Society for Neuro-science 16th Annual Meeting (1986) Abstr. 275.2.
29 S. Einarsson, B. Josefsson, P. Möller and D. Sanchez, Anal. Chem., 59 (1987) 1191-1195.
30 W. Lindner, C. Leitner and G. Uray, J. Chromatogr., 316 (1984) 605-616.
31 C. Pettersson and G. Schill, J. Chromatogr., 204 (1981) 179-183.
32 C.E. Dalgliesh, J. Chem. Soc. (1952) 3940-3942.
33 P. Dayer, T. Leemann, A. Marmy and J. Rosenthaler, Eur. J. Clin. Pharmacol., 28 (1985) 149-153.
34 T. Leemann, P. Dayer and U.A. Meyer, Eur. J. Clin. Pharmacol., 29 (1986) 739-741.
35 W.H. Pirkle, J.M. Finn, J.L. Schreiner and B.C. Hamper, J. Am. Chem. Soc., 103 (1981) 3964-3966.
36 I.W. Wainer, T.D. Doyle, K.H. Donn and J.R. Powell, J. Chromatogr., 306 (1984) 405-411.
37 U.K. Walle, T. Walle, S.A. Bai and L.S. Olanoff, Clin. Pharmacol. Ther., 34 (1983) 718-723.
38 G. Schill, I.W. Wainer and S.A. Barkan, J. Liq. Chromatogr., 9 (1986) 641-666.
39 J. Hermansson, J. Chromatogr., 269 (1983) 71-80.
40 J. Hermansson, J. Chromatogr., 298 (1984) 67-78.
41 J. Hermansson, J. Chromatogr., 325 (1985) 379-384.
42 D.W. Armstrong, T.J. Ward, R.D. Armstrong and T.E. Beesley, Science, 232 (1986) 1132-1135.
43 S. Caccia, C. Chiabrando, P. De Ponte and R. Fanelli, J. Chrom. Sci., 16 (1978) 543-546.
44 S. Caccia, G. Guiso, M. Ballabio and P. De Ponte, J. Chromatogr., 172 (1979) 457-462.
45 W.A. König and K. Ernst, J. Chromatogr., 280 (1983) 135-141.
46 W.A. König, K. Ernst and J. Vessman, J. Chromatogr., 294 (1984) 423-426.

47 O. Gyllenhaal, W.A. König and J. Vessman, J. Chromatogr., 350 (1985) 328-331.
48 G. Gübitz and S. Mihellyes, J. Chromatogr., 314 (1984) 462-466.
49 G. Pflugmann, H. Spahn and E. Mutschler, J. Chromatogr., 416 (1987) 331-339.
50 K. Kawashima, A. Levy and S. Spector, J. Pharmacol. Exp. Ther., 196 (1976) 517-523.
51 L. Wang, M. Chorev, J. Feingers, A. Levitzki and M. Inbar, FEBS Lett., 199 (1986) 173-178.
52 S. Chamat, J. Hoebeke and A.D. Strosberg, J. Immunol., 133 (1984) 1547-1552.
53 D.G. Sawutz, D. Sylvester and C.J. Homcy, J. Immunol., 135 (1985) 2713-2718.
54 T. Walle and U.K. Walle, Res. Commun. Chem. Pathol. Pharmacol., 23 (1979) 453-464.
55 L.S. Olanoff, T. Walle, T.D. Cowart, U.K. Walle, M.J. Oexmann and E.C. Conradi, Clin. Pharmacol. Ther., 40 (1986) 408-414.
56 W.L. Nelson and M.L. Powell, Drug Metab. Dispos., 7 (1979) 351-355.
57 T. Walle, J.E. Oatis, Jr., U.K. Walle and D.R. Knapp, Drug Metab. Dispos., 10 (1982) 122-127.
58 T. Walle, M.J. Wilson, U.K. Walle and S.A. Bai, Drug Metab. Dispos., 11 (1983) 544-549.
59 T. Walle, U.K. Walle, M.J. Wilson, T.C. Fagan and T.E. Gaffney, Brit. J. Clin. Pharmacol., 18 (1984) 741-747.
60 S. Honma, T. Ito and A. Kambegawa, Chem. Pharm. Bull., 33 (1985) 760-768.
61 L.S. Olanoff, T. Walle, U.K. Walle, T.D. Cowart and T.E. Gaffney, Clin. Pharmacol. Ther., 35 (1984) 755-761.
62 U.K. Walle, T. Walle, S.A. Bai and L.S. Olanoff, Clin. Pharmacol. Ther., 34 (1983) 718-723.
63 S.A. Bai, M.J. Wilson, U.K. Walle and T. Walle, J. Pharmacol. Exp. Ther., 227 (1983) 360-364.
64 S.A. Bai, U.K. Walle, M.J. Wilson and T. Walle, Drug Metab. Dispos., 11 (1983) 394-395.
65 T. Walle, Drug Metab. Dispos., 13 (1985) 279-282.
66 W.L. Nelson and M.J. Bartels, J. Labelled Compd. & Radiopharm., 20 (1983) 1135-1142.
67 W.L. Nelson and M.J. Bartels, Drug Metab. Dispos., 12 (1984) 382-384.
68 W.L. Nelson and M.J. Bartels, Drug Metab. Dispos., 12 (1984) 345-352.
69 W.L. Nelson and H.U. Shetty, Drug Metab. Dispos., 14 (1986) 506-508.
70 J.A. Thompson, J.E. Hull and K.J. Norris, Drug Metab. Dispos., 9 (1981) 466-471.
71 B.K. Wilson and J.A. Thompson, Drug Metab. Dispos., 12 (1984) 161-164.
72 S.A. Bai and T. Walle, Drug Metab. Dispos., 12 (1984) 749-754.
73 H. Ehrsson, J. Pharm. Pharmacol., 27 (1975) 971-973.
74 K.H. Wingstrand and T. Walle, J. Chromatogr., 305 (1984) 250-255.
75 A. Rubin, M.P. Knadler, P.P.K. Ho, L.D. Bechtol and R.L. Wolen, J. Pharm. Sci., 74 (1985) 82-84.
76 E. Matsutera, Y. Nobuhara and Y. Nakanishi, J. Chromatogr., 216 (1981) 374-379.
77 V.K. Piotrovskii, Y.A. Zhirkov and V.I. Metelitsa, J. Chromatogr., 309 (1984) 421-425.
78 E.H. Gold, W. Chang, M. Cohen, T. Baum, S. Ehrreich, G. Johnson, N. Prioli and E.J. Sybertz, J. Med. Chem., 25 (1982) 1363-1370.
79 T.J. Cholerton, J.H. Hunt and M. Martin-Smith, J. Chromatogr., 333 (1985) 178-185.

318

SUPPLEMENT 1

RETENTION INDICES OF BETA-BLOCKERS

Retention indices of underivatized beta-blockers

	OV 1 (refs. 1,2)	OV 101 (ref. 3)	SP 2100 (ref. 4)	OV 17 (refs. 1-3)	OV 225 (ref. 4)	SP 1000 (ref. 4)
Acebutolol	2795	2815		3290 3336		
Alprenolol	1840 1816	1826	1834	2044 2076 2078	2462	2523
Atenolol		2538		2882		
Befunolol		2274		2686		
Bucumolol		2526		3002		
Bufetolol		2325		2692		
Bunitrolol	1934	1917	1989	2268 2262	2901	2924
Bunolol	2353			2644		
Bupranolol		1925	1939	2177 2188	2618	2653
Carteolol		2555		3045		
Exaprolol	2619			2379		
Indenolol		1977		2274		
Metipranolol	2274		2255	2520	3113	3148
Metoprolol	2041 2052	2022	2051	2294 2336 2323	2794	2871

(continued)

	OV 1 (refs. 1,2)	OV 101 (ref. 3)	SP 2100 (ref. 4)	OV 17 (refs. 1-3)	OV 225 (ref. 4)	SP 1000 (ref. 4)
Nadolol	2631			3031		
Nifenalol			1953		2852	2897
Oxprenolol	1929 1989	1900	1934	2169 2189 2201	2625	2691
Pindolol	2245 2253	2234		2605 2697 2655		
Propranolol	2152 2170	2162	2180	2417 2497 2483	3013	3096
Sotalol	2413			2839		
Timolol	2309	2285	2306	2651 2594	3182	3206
Toliprolol	1721 1691		1745	1939 1980	2365	2435

Retention indices of acylated beta-blockers

	A[a]	TFA[b]			HFB[c]	
	OV 101 (ref. 5)	OV 1 (ref. 2)	OV 101 (ref. 3)	OV 17 (refs. 2,3)	OV 1 (ref. 2)	OV 17 (ref. 2)
Alprenolol	2275	1861	1860	1975 1976	1861	1892
Atenolol	2380		2133	2400		
Befunolol			2251	2522		
Betaxolol	2680					
Bucumolol			2215	2649		
Bufetalol			2329	2391		
Bunitrolol	2070		1976	2084		
Bunolol	2460	2088		2431	2111	2390
Bupranolol	2370		1963	2085		
Carazolol	2810					
Carteolol	2700		2208	2754		
Exaprolol		2170		2293	2160	2179
Indenolol			1998	2159		
Labetalol	3400					
Mepindolol	2390					
Metipranolol	2670	1990		2073	2060	1984
Metoprolol	2480	2045	2049	2202	2024	2107

(continued)

	A[a]	TFA[b]			HFB[c]	
	OV 101 (ref. 5)	OV 1 (ref. 2)	OV 101 (ref. 3)	OV 17 (refs. 2,3)	OV 1 (ref. 2)	OV 17 (ref. 2)
Nadolol	2650					
Nifenalol	2305					
Oxprenolol	2390	1930	1931	2074 2073	1928	1989
Pindolol	2240		2116	2248	2148	2104
Propranolol	2330	2166	2139	2309 2349	2133	2209
Sotalol	2675					
Timolol	2290					
Toliprolol	2230	1728		1858	1748	1769

[a] Acetylated

[b] Trifluoroacetylated

[c] Heptafluorobutyrated

Retention indices of boronated beta-blockers

	OV 17		
	BB[a]	PB[b]	DCPB[c]
	(ref. 6)		(ref. 7)
Alprenolol	2303	2749	3046
Atenolol			3870
Bufetalol	2935	3408	
Bupranolol	2455	2870	
Carteolol	3275	3759	
Metoprolol			3346
Nadolol	3171	4167	
Oxprenolol	2426	2876	3200
Pamatolol			3568
Pindolol	2896	3447	
Practolol			3770
Propranolol	2706	3238	3535
Timolol			3660

[a] n-Butylboronated
[b] Phenylboronated
[c] 2,4-Dichlorobenzeneboronated

REFERENCES

1   T. Daldrup, F. Susanto and P. Michalke, Fresenius Z. Anal.
    Chem., 308 (1981) 413-427.
2   V. Marko, Chem. Papers, 42 (1988) 375-380.
3   A. Yamaji, K. Kataoka, N. Kanamori, M. Oishi and E. Hiraoka,
    Yakugaku Zasshi, 105 (1985) 1179-1183.
4   L. v. Meyer, G. Drasch, G. Kaukert, L. Riedl and A. Riedl,
    Beitr. Ger. Med., 37 (1979) 363-366.
5   H. Maurer and K. Pfleger, J. Chromatogr., 382 (1986) 147-165.
6   T. Yamaguchi, Y. Morimoto, Y. Sekine and M. Hashimoto, J.
    Chromatogr., 239 (1982) 609-615.
7   C.F. Poole, L. Johansson and J. Vessman, J. Chromatogr., 194
    (1980) 365-377.

SUPPLEMENT 2

STRUCTURES OF BETA-BLOCKERS
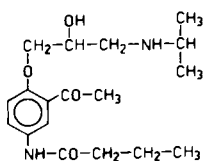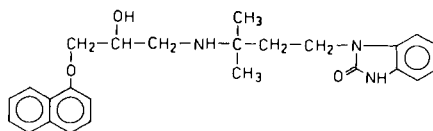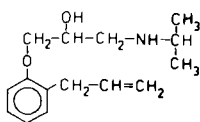
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| |
OH   CH₃
                CH₃

O-CO-CH₃

NH-CO-CH₂-CH₂-CH₃

Acebutolol

CH₂-CH-CH₂-NH-C-CH₂-CH₂-N
| |
OH   CH₃
              CH₃

O=

Adimolol

CH₂-CH-CH₂-NH-CH
| |
OH   CH₃
                CH₃

O
CH₂-CH=CH₂

Alprenolol

CH₂-CH-CH₂-NH-CH
| |
OH   CH₃
                CH₃

O

CH₂-CO-NH₂

Atenolol

CH₂-CH-CH₂-NH-CH
| |
OH   CH₃
                CH₃

H₃C-CO
O

Befunolol

CH₂-CH-CH₂-NH-CH
| |
OH   CH₃
                CH₃

O

CH-CH₂-CH₂-CH₂-O-CH₃
|
CH₂

Betaxolol

CH₂-CH-CH₂-NH-CH₂-CH₂
|
OH

O-CH₃

O-CH₃

CH₃

Bevantolol

CH₂-CH-CH₂-NH-CH
| |
OH   CH₃
                CH₃

O

CH₂-O-CH₂-CH₂-O-CH
                     |
                     CH₃

Bisoprolol

O-CO

CH₂-CH-CH₂-NH-C-CH₃
| |
O   CH₃
              CH₃

H₃C   NH

Bopindolol

CH₂-CH-CH₂-NH-CH
| |
OH   CH₃
                CH₃

O
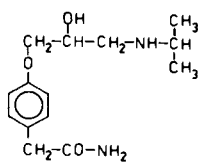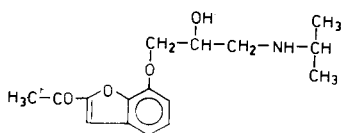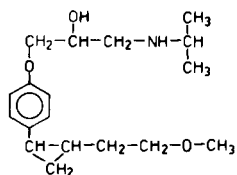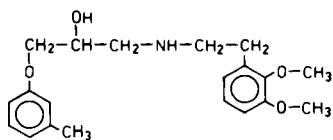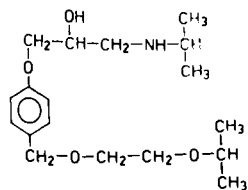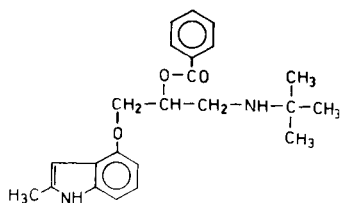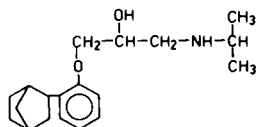
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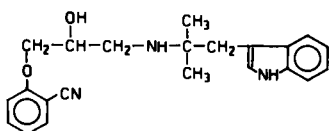
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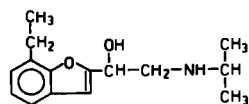
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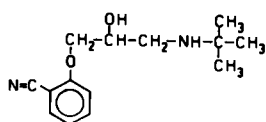
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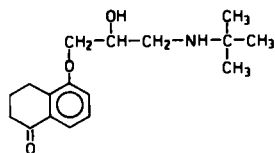
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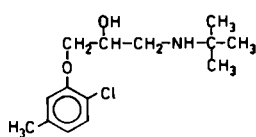
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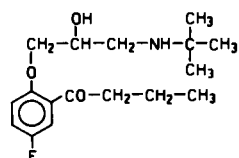
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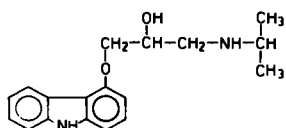
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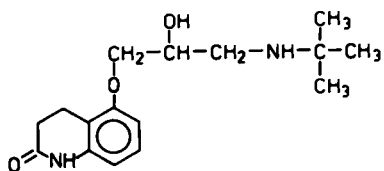
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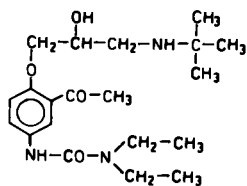
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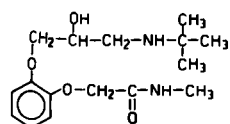
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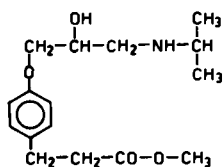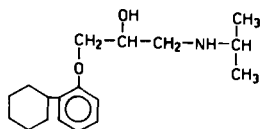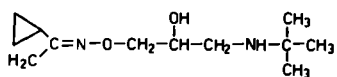
OH   CH₃
| |
CH₂-CH-CH₂-NH-CH
| |
O   CH₃

CH₂-CH₂-CO-O-CH₃

Esmolol

OH   CH₃
| |
CH₂-CH-CH₂-NH-CH
| |
O   CH₃

Exaprolol

OH
|
C=N-O-CH₂-CH-CH₂-NH-C-CH₃
| |
H₂C   CH₃

Falintolol

OH   CH₃
| |
CH₂-CH-CH₂-NH-C-CH₃
| |
O   CH₃
|
F

CH₂-CH₂-CO-O-CH₂-CH₃

Flumolol

OH   CH₃
| |
CH₂-CH-CH₂-NH-CH
| |
O   CH₃

Indenolol

OH   CH₃
| |
CH-CH₂-NH-CH
H₂N-OC |
           CH₂-CH₂
HO

Labetalol

OH   CH₃
| |
CH₂-CH-CH₂-NH-CH
| |
O   CH₃

H₃C-N

Mepindolol

OH   CH₃
| |
CH₂-CH-CH₂-NH-CH
| |
O   CH₃

H₃C

H₃C   CH₃
|
O
|
CO-CH₃

Metipranolol

OH   CH₃
| |
CH₂-CH-CH₂-NH-C-CH₃
| |
O   CH₃

CH₂-CH₂-O-CH₃

Metoprolol

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| |
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| |
O   CH₃
|
CO-CH₃
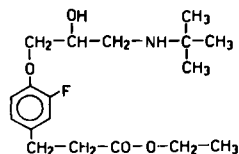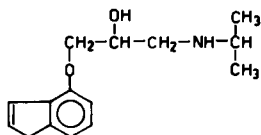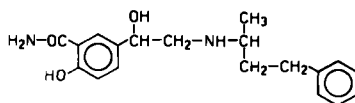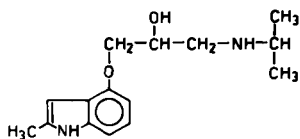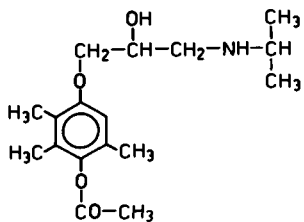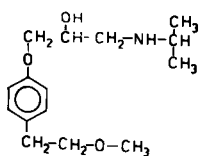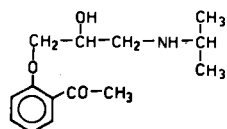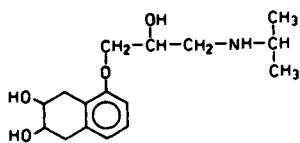
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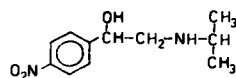
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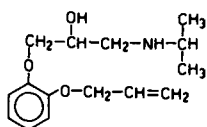
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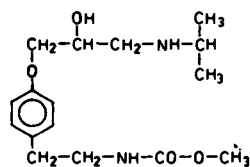
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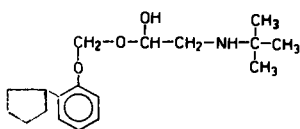
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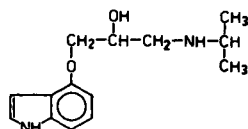
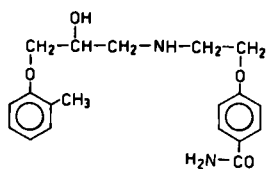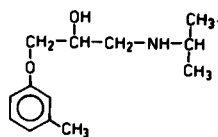Pindolol
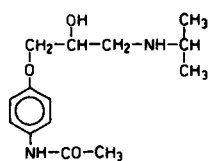
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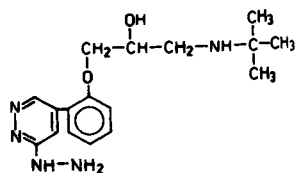
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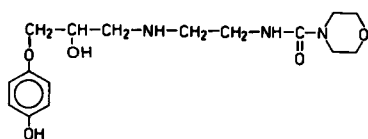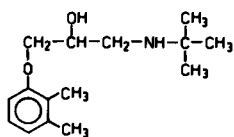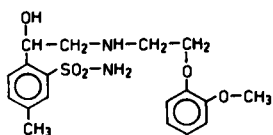
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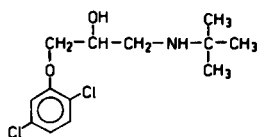
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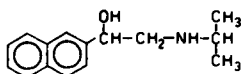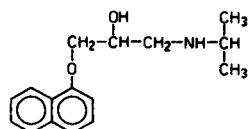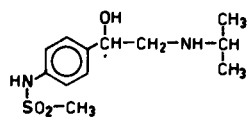
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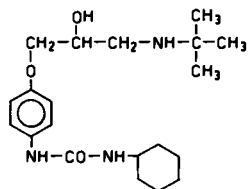
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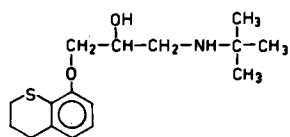
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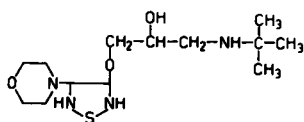
Timolol

Tolamolol

Toliprolol

Xamoterol

Xibenolol

YM-09538

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